Universidade do Minho
Escola de Engenharia

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Doctoral Dissertation for PhD degree in Chemical and Biological Engineering

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Título da tese

Development of Fermentation Process for Fungal Fucoidanases Production

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To Monita
and Vichita

"We are the Dancing Queens"

SUMMARY

This thesis is focused in the development of an integral bioprocess for fungal fucoidanases production, in order to develop new strategies of fermentative conditions and parameters. In the recent years, the interest in the research field of fucoidans and fucoidanases has strongly increased due to the high potential in medical exploitation of fucoidans and its degradation products.

Fucoidans are highly sulfated polysaccharides of brown algae widely used in fields as food and beverage, pharmacy, health medicine and cosmetics, due to their important biological properties as antitumoral, anticoagulant and antiviral activities. Enzymes with known specificities that catalyze the degradation of fucoidan are important tool for studying the relation between structure and biological role of this class of polysaccharide.

Firstly, *Aspergillus niger* PSH, *Mucor sp*. 3P, and *Penicillium purpurogenum* GH2 were selected among several fungal strains, isolated from Northeast Mexican desert, for their fucoidan hydrolyzing ability, by measuring the strains kinetic and morphometric behavior over plate assays containing fucoidan as target polysaccharide and testing different nitrogen sources. Furthermore, submerged fermentations testing the synergy of fucoidan with others sugars for inducing high enzyme titles showed that *A. niger* PSH synthesized the highest titles of sulfated fucan-degrading enzymes with the culture media of fucoidan-sucrose.

Secondly, *Fucus vesiculosus* algae were collected at Praia Norte, Viana de Castelo, Portugal, for hydrothermal extraction of sulfated polysaccharides (fucoidan) using microwave and autohydrolysis processes. Experimental designs were applied evaluating different conditions of temperature/ pressure, time and alga/water ratio to establish a condition to maximize the extraction results. For microwave assisted extraction at 120 psi, 1 min and 1:25 alga:water rate was the best condition for the fucoidan recovery (18%); whereas the extraction by autohydrolysis showed optimal yield at 180 °C for 20 min (16.5% w/w). The obtained products were characterized and it was verified the presence of fucose as the main constituent of these polysaccharides and a SO_3 content

higher than 20%. Moreover, as fucoidan is said to possess so many interesting bioactivities, the extracted products were analyzed for antioxidant activity, displaying a strong free radical scavenging effect; this behavior is possibly associated with the high content of sulphate groups.

Finally, the solid stated fermentation (SSF) system was assessed for the induction of fucoidanases using the selected fungal strains and the *Fucus vesiculosus* algae, obtained after both hydrothermal extraction. A rotational drum bioreactor was designed to carry out the culture experiments. The results showed that the maximum fucoidanase activity was obtained with *Mucor sp.* 3P and algae after autohydrolysis pre-treatment production (3.82 U L^{-1}); also the mixture of the solids showed to influence in the induction of the enzyme production. Moreover, a SSF scale up experiments showed that controlling the moisture content and the addition of inert support on algae substrates increment the enzyme production (9.62 U L^{-1}). These results indicate, that the fucoidan hydrolytic enzyme can be extracellular, induced by a solid substrate rich of fucose and fucoidan.

In general, with the established and developed methods of this thesis was possible to determine the solid state fermentation parameters for fucoidan hydrolytic enzyme production using terrestrial fungal strains and also allowed to set up the extraction conditions and physicochemical characteristics for the production of sulfated fucans by environmental friendly process. This opens up new vistas to modify fucoidan and to develop the postulated bioactive potentials.

RESUMO

Esta tese foca-se no desenvolvimento de um bioprocesso integral para a produção de fucoidanases fúngicas, a fim de desenvolver novas estratégias de condições fermentativas e parâmetros. Nos últimos anos, o interesse na área de pesquisa de fucoidanos e fucoidanases aumentou significativamente devido ao elevado potencial na exploração médica de fucoidanos e dos seus produtos de degradação.

Os fucoidanos são polissacarídeos de algas castanhas, altamente sulfatados, bastante usados em áreas como a dos alimentos e bebidas, farmacêutica, médica e cosmética, devido às suas importantes propriedades biológicas como as suas actividades antitumoral, anticoagulante e antiviral. Enzimas com especificidades conhecidas que catalisam a degradação de fucoidano são uma ferramenta importante para o estudo da relação entre a estrutura e papel biológico desta classe de polissacarídeos.

Numa primeira fase, *Aspergillus niger* PSH, *Mucor sp.* 3P e *Penicillium purpurogenum* GH2 foram seleccionadas entre várias estirpes de fungos isoladas do deserto do Nordeste do México, devido à sua capacidade de hidrolisar fucoidano, através da medição do comportamento cinético e morfométrico das estirpes em placas de ensaio contendo fucoidano como polissacarídeo alvo e testando diferentes fontes de azoto. Além disso, as fermentações submersas testando a sinergia de fucoidano com outros açúcares para indução de elevadas actividades enzimáticas evidenciaram que *A. niger* PSH sintetizou a mais elevada actividade de enzimas que degradam fucanos sulfatados com o meio de cultura de fucoidano-sucrose.

Numa segunda fase, algas *Fucus vesiculosus* foram recolhidas na Praia Norte, Viana do Castelo, Portugal, para extracção hidrotermal dos polissacarídeos sulfatados (fucoidanos) usando processos de microondas e auto-hidrólise. Foram aplicados desenhos experimentais para avaliar diferentes condições de temperatura/pressão, tempo e razão alga/água para estabelecer uma condição para maximizar os resultados de extracção. Na extracção assistida por microondas, 120 psi, 1 min e uma razão de 1:25 água:água, foram as melhores condições para a recuperação de fucoidano (18%); enquanto que a extracção por auto-hidrólise revelou um rendimento óptimo a 180 ºC

durante 20 minutos (16.5% w/w). Os produtos obtidos foram caracterizados e verificou-se a presença de fucose como o principal constituinte desses polissacarídeos e um conteúdo em SO3 superior a 20%. Além disso, como se diz que o fucoidano possui tantas bioactividades interessantes, os produtos extraídos foram analisados a nível de actividade antioxidante, exibindo um forte efeito na eliminação de radicais livres; este comportamento está possivelmente associado ao elevado conteúdo em grupos sulfato.

Finalmente, o sistema de fermentação em estado sólido (FES) foi avaliado para a indução de fucoidanases utilizando as estirpes fúngicas seleccionadas e as algas Fucus vesiculosus, obtidas após extracção hidrotermal. Foi desenhado um bioreactor de tambor rotativo para realizar os ensaios de cultura. Os resultados demonstraram que a actividade máxima de fucoidano foi obtida com Mucor sp. 3P e com algas após produção com pré-tratamento de auto-hidrólise (3.82 U L^{-1}); a mistura dos sólidos também demonstrou a influência na indução da produção da enzima. Além disso, as experiências de aumento de escala de FES mostraram que, controlando o teor de humidade, a adição do suporte inerte em substratos de algas aumenta a produção de enzima (9.62 U L^{-1}). Estes resultados indicam que a enzima que hidrolisa o fucoidano pode ser extracelular, induzida por um substrato sólido rico em fucose e fucoidano.

De um modo geral, com os métodos estabelecidos e desenvolvidos nesta tese foi possível determinar os parâmetros de fermentação em estado sólido para produção de enzimas que hidrolisam o fucoidano usando estirpes de fungos terrestres e também foi possível estabelecer as condições de extracção e as características físico-químicas para a produção de fucanos sulfatados por um processo amigo do ambiente. Isto abre novas vistas para modificar o fucoidano e desenvolver os potenciais bioactivos postulados.

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Ac	Sodium acetate
$ABTS^{\bullet+}$	2,2-azino-bis3-ethylbenzothiazoline-6-sulfonic acid (radical-scavenging)
AD	Alga degradation
AH	Autohydrolysis
ANOVA	Analysis of variance
ASF	Autohydrolyzed sulfated polysaccharides.
a_w	Water activity
BHA	Butylated hydroxyanisole
CCD	Central composite design
CZ	Czapek media
ΔC:	Cool rate
D_h	Hyphal diameter
DNS	3,5-dinitrosalicylic acid method
$DPPH^{\bullet}$	1,1-Diphenyl-2-picryl-hydrazyl (radical-scavenging)
DPV	Differential pulse voltammetry
DSC	Diffential scanning calorimetry
EE	Effect estimates
Glu	Glucose
FRAP	Ferric reducing capacity
Fru	Fructose
FTIR	Fourier transform infrared

Fuc	Fucoidan
HA	Autohydrolyzed alga
HPLC	High performance liquid chromatography
ΔH	Heat rate
H_2O_2	Hydrogen peroxide
Lac	Lactose
L_{av}	Hyphal length
LOI	Lipid oxidation
MA	Microwave alga
MAE	Microwave assisted extraction
MM	Minimal media
MSF	Microwave sulfated polysaccharides
NHA	Non isothermal autohydrolysis alga
$O_2^{-\bullet}$	Superoxide anions
$^{\bullet}OH$	Hydroxyl radicals
P_R	Enzyme productivity
p	Level of significance
R^2	Coefficient of determination
R_0	Severity factor
RI	Refractive index
ROS	Reactive oxygen species
SE	Standard error
SEM	Scanning electron microscopy

SmF	Submerged fermentation
SO_3	Sulfate
SE	Standard errors
SHA	Non-isothermal autohydrolyzed alga supported on synthetic fiber
SSF	Solid state fermentation
Suc	Sucrose
TGA	Thermogravimetric analysis
TS	Total sugar
TS_L	Total sugars in the liquor after extraction
U	Enzymatic unit
UA	Untreated alga
U_fA	Fresh untreated algae
U_r	Radial growth rate
WA	Alga weight
Y_i	Dependent variables
$Y_{E/X}$	Enzyme yield
$Y_{X/S}$	Biomass yield
x_i	Independent variables
μ	Specific growth rate

,

CHAPTER 1

MOTIVATION AND OUTLINE

ABSTRACT

This chapter introduces the thesis motivation for the study of fucoidans and fucoidanases with a special focus in the development of a whole process to produce fucoidan hydrolytic enzymes by filamentous fungi. Finally, the thesis outline is explained.

1.1 THESIS MOTIVATION

Marine ecosystem is a rather unexplored source of natural substances with enormous bioactive potential having attracted the attention of biologist and chemists around the world for the last fifty years.

Although the first report on fucoidan isolation from brown seaweed was about 90 years ago[Ω], only after 1970's a growing interest in this research field started to occur, as a consequence of the interest in the biological activities of this compounds, such as anti-viral, anti-bacterial, anti-coagulant and anti-tumor.

Fucoidanases are very complex enzymes due to the large variety of fucoidan structure, and its catalytic specificities influence directly with the different bioactivities fuctions of sulfated polysaccharides. Non aggressive recovery of oligomers of sulfated polysaccharides may be obtained by fucoidanase enzymolysis, without removal of its side substitute groups, having as final objective the improvement of its properties. Moreover, the high variety of fucoidan sources requires a large number of cleaving patterns, been impossible until now to describe a "unique fucoidanase", but only a group of hydrolyzing enzymes with exact cleaving mechanism still unknown.

The production of fucoidan-degrading enzymes has advanced quite slowly, the majority of the results being reported in the last decade and corresponding to the screening and isolation of organism (invertebrates, marine bacteria and marine fungi) able to degrade fucoidan. Only a few have been able to isolate the corresponding enzyme and to elucidate its structure. Currently, *Saccharophagus degradans* is the only commercially available microorganism with fucoidan-degrading ability, and there is not any information about commercially available endofucanase. Information about fermentation conditions for fucoidanase production is also scarce, and is based only in marine fungus and bacteria.

[Ω] *Kylin H (1918) Biochemistry of seaweeds. Z Physiol Chem 101:236–247*

In order to understand the biodegradation process of fucoidan, it is necessary the presence of specific enzymes that participate in its hydrolytic mechanisms. Fucoidanases can be produced by biotechnology ways through microbial cultures like submerged and solid fermentation systems. The potential of solid state fermentation (SSF) has been recognized for the last 20 years, due to some advantages that this system presents over conventional submerged cultures, including higher volumetric productivity, relatively higher concentration of the products and lesser effluent of crops; for the recovery of value-added products such as biologically active secondary metabolites.

1.2 THESIS OUTLINE

The main purpose of this thesis was the development of a process for fucoidanases production involving the screening of fungal strains, green technologies to recovery fucoidan, and non-traditional fermentative methods to induce fucoidan hydrolytic enzymes. Thus, the aims of this thesis were:

1- to identify fungal strains able to grow over fucoidan-based media and induce active fucoidanases (Figure 1-red labels)

2- to establish the optimal conditions for the extraction of sulfated polysaccharides (fucoidan) from *Fucus vesiculosus* seaweed by microwave assisted and autohydrolysis processes (Figure 1-green labels)

3- to evaluate the antioxidant activity potential of *Fucus vesiculosus* fucoidan recovered by hydrothemal conditions (Figure 1-green labels)

4- to determine the solid state fermentation conditions using algae substrates as inducers in order to produce fucoidan-hydrolyzing enzymes (Figure 1-blue labels)

Based on the objectives stated above, this thesis was organized in eight chapters. The main topics addressed are introduced in this chapter, while Chapter 2 provides a literature review, Chapters 3 to 7 contain the main experimental results while Chapter 8 presents the main conclusions and recommendations for future work.

In ***Chapter 1*** the subject of this thesis and its relevance in the context of fucoidan and fucoidanase study is introduced

An overview of the biotechnology processes related to the production of fucoidanases, specifically by solid state fermentation, including the current knowledge in fucoidan characterization and its biochemical importance, is reviewed in ***Chapter 2***.

In ***Chapter 3***, a screening of fungal strains by the quantification of kinetic and morphology features able to growth over fucoidan- agar-plate media, is investigated. Additionally, a preliminary study about the effect of different saline media and carbon sources as enzyme inducers in submerged fermentations is evaluated.

The extraction and characterization of fucoidan from *Fucus vesiculosus* seaweed by microwave assisted extraction (MAE) are described in ***Chapter 4***. The influence of pressure, extraction time and alga/water ratio on the extraction yields are presented.

In ***Chapter 5***, the autohydrolysis (AH) process optimization for fucoidan production by response surface methodology extraction is presented. The effects of the temperature and reaction time on the fucoidan extraction yield, and the physic-chemical characteristics of the extracted fucoidan are described.

The influence of different hydrothemal extraction (MAE and AH) processes on the antioxidant potential of the recovered sulfated polysaccharides (fucoidan) is evaluated in ***Chapter 6***.

In ***Chapter 7***, the potential of solid state fermentation in the induction of fucoidanases by fungal strains is assessed. Experiments were done in a rotational drum bioreactor at laboratory scale using pre-treated algae as culture substrate. The scale up potential for enzyme production is discussed.

Finally, ***Chapter 8*** summarizes the main conclusions obtained from the work described in this thesis, and perspectives for further research are proposed.

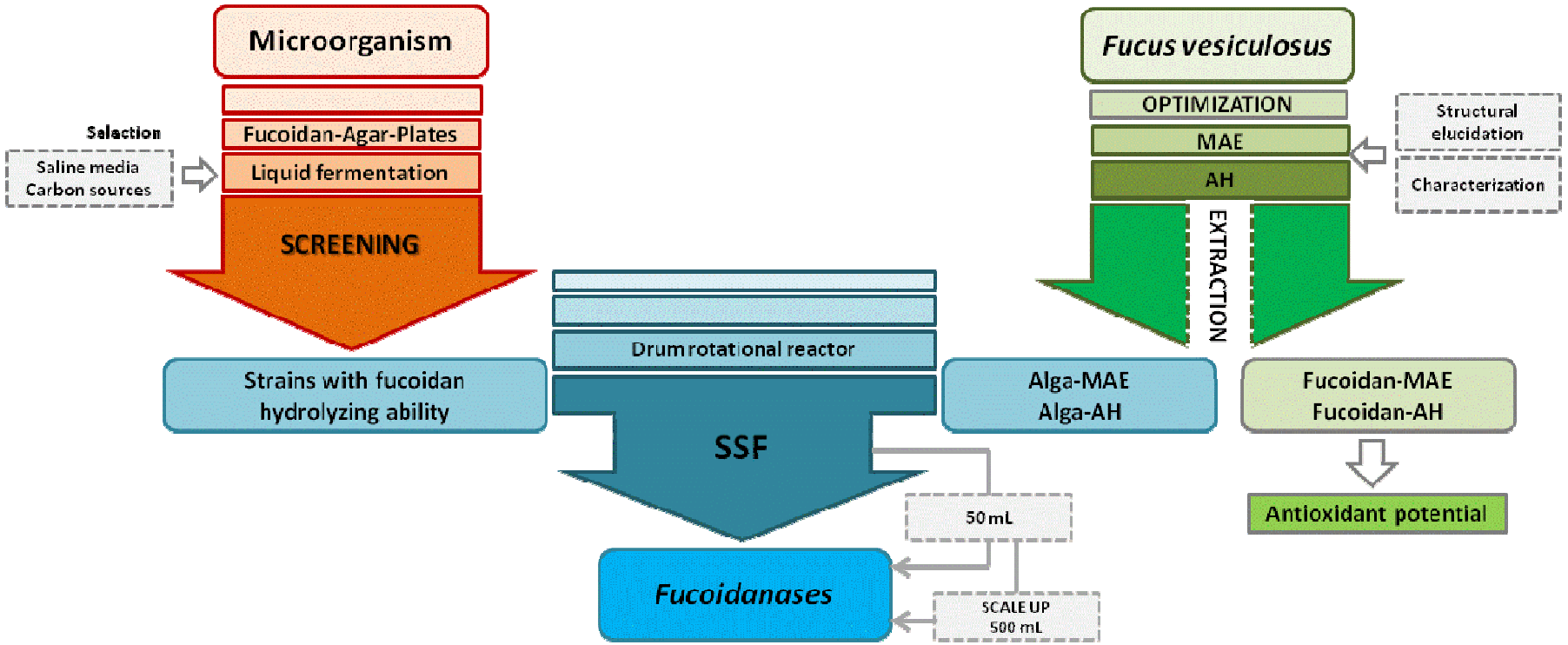

Figure 1. Thesis set up

CHAPTER 2

INTEGRAL BIOPROCESS FOR ENZYME PRODUCTION USING SOLID STATE FERMENTATION AND SEAWEED AS SUBSTRATE AND SUPPORT

ABSTRACT

There is a considerable interest in exploring marine habitats for the production of biologically active secondary metabolites and the development of new biotechnology processes. Solid-state fermentation (SSF) is a potential technology for the conversion of biomass marine such as fucoidan from seaweed into high added value products. This chapter is focused on the description of SSF and its fundaments together with the most relevant information on seaweed use, fucoidan applications and fucoidanase production.

2.1 INTRODUCTION

Biotechnology is the use of biological processes, organisms, or systems to manufacture products intended to improve the quality of human life through its impact on agriculture, marine, animal husbandry, health, environmental protection, material transformation, fine chemical, food processing, pharmaceutical and other areas. The rapid development of biotechnology has impacted diverse sectors of the economy over the last several years. In order for current biotechnology research to continue revolutionizing industries, new processes must be developed to transform current research into viable market products (Pandey, 2004; Gavrilescu and Chisti, 2005). For example, brewing is regarded as a typical example of traditional or old biotechnology, because of its long history (Linko *et al.*, 1998; T.H. Gadaga *et al.*, 1999)), that can benefit from biotechnology research.

Actually, marine biotechnology, understood as the application of modern biotechnology to marine processes, is an area of significant industrial importance whose ramifications will reach almost every major industrial sector including health, environment, energy, food, chemicals (Figure 2.1) (Zaborsky, 1999).

Bioengineering integrates physical, chemical, mathematical and engineering principals to study biology and medicine. It advances fundamental concepts; creates knowledge from the molecular to the organ systems levels; and develops innovative biologics, materials, and bioprocesses. Currently, extensive application of bioprocesses has created an environment for many researchers to expand knowledge of and interest in biotechnology. Finally, application of biochemical engineering in biotechnology has become a new way of making commercial products (Vogel and Torado, 1997; Citron and Nerem, 2004; Najafpour, 2007).

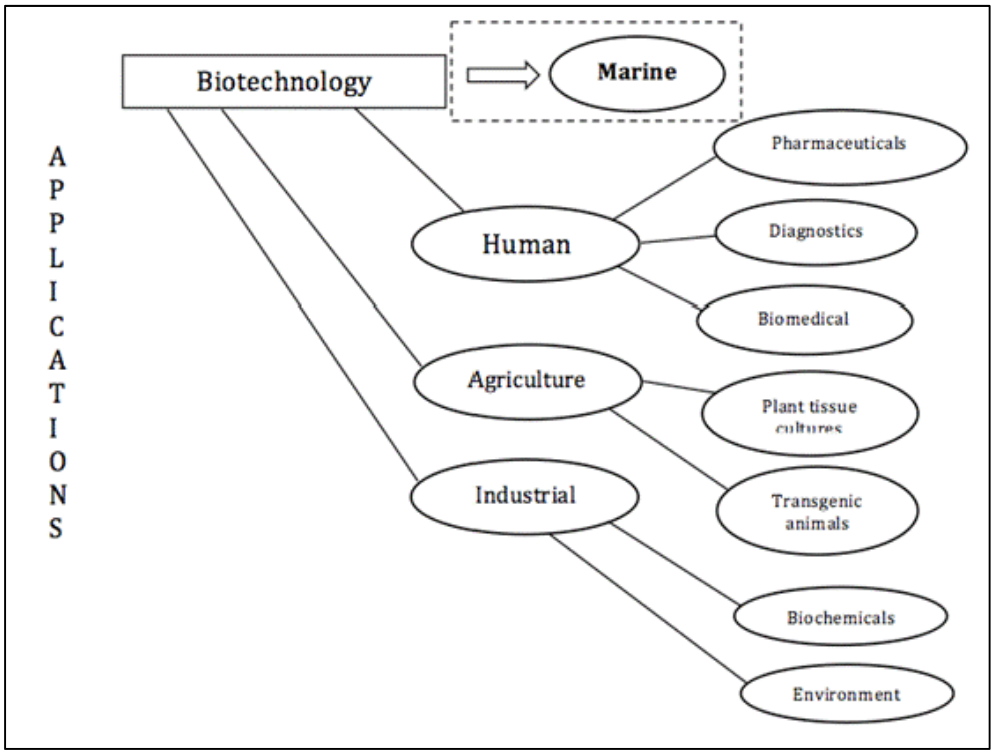

Figure 2.1.Applications of biotechnology in various market sectors

2.2 BIOPROCESS FERMENTATION

Fermentation dates back to prehistoric times when cheese was made in Iraq (6000 BC). Egyptians first discovered the use of yeast to make leavened bread and wine (4000 BC); Sumerians fermented barley to beer (1750 BC); and Chinese used moldy soybean curds as antibiotics (500 BC) (Aiba, *et al.*, 1973; Ross *et al.*, 2002; Bamforth, 2005). The first half of the twentieth century saw the development of fermentation processes for the industrial production, such as fuels (ethanol, butanol) organic acids (acetic acid, lactic acid, citric acid) and other chemicals were produced from biomass (Willke and Vorlop, 2004). The period of 1940's has been termed as the 'Golden Era' of fermentation industry, which saw the dramatic discovery and development of the wonder penicillin process during the Second World War that reaffirmed the development of industrial fermentation. Application of biological sciences in industrial processes is known as bioprocessing. Nowadays most biological and pharmaceutical products are produced in

well-defined industrial bioprocesses (Friedkin, 1995; Burns M *et al.*, 2002; Hulse, 2004; Flickinger, 2010).

The fermentation process involves the growth of microorganism and formation of products under different operational conditions that provide the environment for cell growth and survival and product formation. Presently, fermentation technology has gained increased importance due to the power of microbe design biotechnology, perception of its hazards and the risks/benefits of chemical synthesis, together with better economics from the use of renewable raw materials (Sanchez and Demain, 2002; Najafpour, 2007).

The fermentation process is mainly divided into two broad categories: submerged fermentation (SmF) and solid-state fermentation (SSF). The major difference between these two bioprocesses is the amount of free liquid in the substrate. The former has been readily employed in industries for large-scale production of alcohol, organic acids, enzymes, antibiotics, vitamins, and amino acids (Hang and Woodams, 1986; Solís-Pereira *et al.*, 1993; Pastrana *et al.*, 1995; Afifi *et al.*, 2011).

SmF is the most popularly used technique for the production of a large number of products using a wide range of microorganisms. The medium used for submerged fermentation contains relatively highly processed ingredients. The water activity of the medium is high, making it prone to contamination if asepsis is not maintained. Rheological problems can be encountered at high substrate concentrations. Mass transfer from gas to liquid phase is usually a limiting factor, but the use of efficient mixing reduces diffusional limitation of nutrients in SmF. Better bioprocess control of fermentation process is possible with the help of online sensors (Christensen and Marcher, 1996; Pandey *et al.*, 2000; Romero-Gómez *et al.*, 2000; Papagianni, 2004; Khairnar *et al.*, 2007). There is, however a significant interest in using solid-state fermentation techniques to produce a wide variety of second metabolites as enzymes (Robinson *et al.*, 2001). A short review on the development of SSF is given below.

2.2.1 SOLID STATE FERMENTATION

The history of SSF started in Asia on cheese making by *Penicillium rouquefortii*, fermentation of rice by *Aspergillus oryzae* to initiate the koji process. Furthermore, in China, SSF has been used extensively to produce Chinese wine soy sauce and vinegar (Raimbault, 1998; Couto and Sanromán, 2006). During 1960-1970, it was reported the production of mycotoxins by SSF (Krishana, 2005). The current decade has witnessed an unprecedented spurt in SSF for the development of bioprocesses such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop-residues for nutritional enrichment, biopulping, and production of value-added products such as biologically active secondary metabolites from fungi, (Srinophakun and Srinophakhun, 1998; Papagianni *et al.*, 1999; Brand *et al.*, 2000; Castilho *et al.*, 2000; Aguilar *et al.*, 2001; Chen *et al.*, 2002; Suryanarayan, 2003; Viniegra-González *et al.*, 2003; Barrios-González *et al.*, 2005; Kadam *et al.*, 2011; Yalemtesfa, *et al.*, 2010; Madeira Jr. *et al.*, 2011).

Solid-state fermentation is defined as the growth of the microorganisms on moist solid material in the absence or near absence of free water. Microorganisms use the water from the moisture held within the substrate particles (Pandey, 2003; Raghavarao *et al.*, 2003; Bellon-Maurel *et al.*, 2003; Ruiz, 2004). Holker and Lenz, (2005) showed the schematic of some of the microscale process that occur during solid-state fermentation (Figure 2.2). In this process, fungi grow by forming a mycelial mat on the surface of the particles that include the solid substrate. Aerial hyphae protrude into the gaseous space and penetrative hyphae grow into liquid-filled pores. Near the substrate surface and within the pores are the regions of higher metabolic activities. However, the regions of aerial hyphae also show metabolism and there can be a transport of substances from the penetrative to the aerial hyphae. By producing hydrolytic enzymes that diffuse into the solid matrix, fungi can degrade macromolecules into smaller units which are taken up by mycelium to serve as nutrients.

The materials used in SSF can be divided into two categories: inert (synthetic materials) and non-inert (organic materials). The use of support-substrates presents several

advantages, such as the reduction in production costs, since some nutritive substances are also supplied to the microorganisms. The recent studies on SSF process have explored the application of a variety of substrates varying from agro-residues to waste.

SSF on an inert support involves the cultivation of microorganisms on an inert support impregnated with a liquid medium. In this SSF, the inert support serves not only as reservoir of nutrients (in a liquid phase), but also as an anchor point for the microorganisms (Zhu *et al.* 1994; Ooijkaas *et al.* 2000; Orzua *et al.*, 2009). Ooijkaas *et al.* (2000) give, as an example, the cost calculation for spore production of *C. minitans* on chemically defined media on inert support, reporting that fermentation costs account were less than 20% of the production costs.

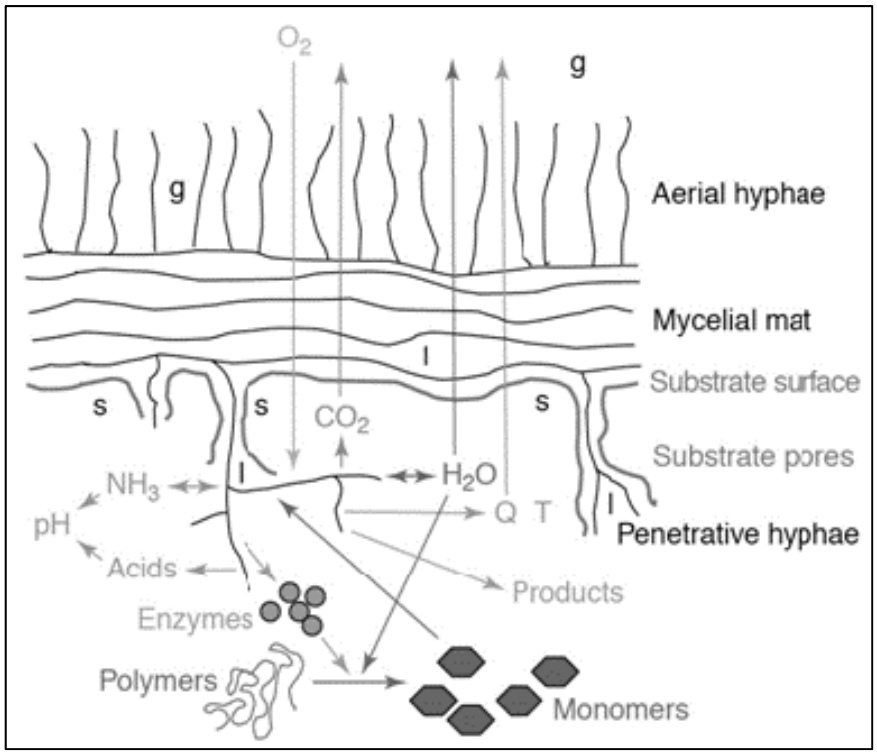

Figure 2.2. A scheme of some microscale processes that occur during solid-state fermentation (adapted from Holker and Lenz, 2005).

Thus, though historically known since centuries, SSF has gained attention from researchers and industries all over the world in recent years, mainly due to the major advantages offered over liquid (submerged) fermentation (SmF). Viniegra-González *et al.* (2003) developed a general approach for the comparison of productivity of enzymes

employing SSF and SmF, trying to explain the reason for the higher production in SSF. Higher biomass, high enzyme production and lower protein breakdown contribute to the better production in SSF. Saqib *et al.* (2010) reported that *Aspergillus fumigatus* produced more protein and a higher specific activity of endoglucanase in SSF than in SmF. Volke-Sepúlveda *et al.* (2003) studied the biodegradation and mineralisation of hexadecane by *Aspergillus niger* in SmF and SSF and reported that the hexadecane consumption was three times higher and fungal growth was up to 30 times faster in SSF than in SmF. Aguilar *et al.* (2002), showed that the use of SSF for fungal protease production allowed high production and more stable enzyme titers than SmF system. In an interesting study, Castilho *et al.* (2000) reported an economic analysis in the production of *Penicillium restrictum* lipase in both SmF and SSF and showed that the total capital investment needed for the SmF process is 78 % higher than that needed for SSF process. Taragano and Pilosof (1999), reported that the production of pectinlyase was 3 times higher in SSF than in SmF. Several industrially important products have been produced by SSF using different supports and substrates (Table 2.1).

There are several important factors that have a decisive impact on the success of a particular technology and need to be considered in its development. Relevant factors in SSF are the selection of microorganism and substrate, optimum process parameters and also the end product.

2.2.2 MICROORGANISM

Many microorganisms are capable of growing on solid substrates, but only filamentous fungi can grow to a significant extent in the absence of free water. SSF closely resembles the natural way of life of filamentous fungi. Fungi are adapted to life on specific natural substrates, thus host specificity is a primary consideration in SSF. Filamentous fungi comprise an industrially very important sub-group, since they are used for the production of a wide variety of products ranging from primary and secondary metabolites and further on to industrial enzymes (Tendergy and Szakacs, 2003; Pandey, 2003; Holker *et al.*, 2004).

Table 2.1. Production of different metabolites using SSF

Microorganism	**Substrate**	**Product**	**Reference**
Aspergillus niger FGSA733	*Jatropha curcas* seed	Xylanase and cellulase	Ncube *et al.* (2012)
Pleurotus ostreatus	Potato dextrose yeast extract medium/ polyurethane foam (as support)	Laccase	Mazumder *et al.* (2009)
Rhizopus strains	Cassava bagasse, apple pomace	Volatile carbons as flavours, acetaldehyde, ethanol, propanol, esters.	Christen *et al.* (2000)
Aspergillus niger	Sugarcane bagasse, coffee husk, cassava bagasse.	Citric acid	Vandenberghe *et al.* (2000)
Bacillus subtilis	Wheat bran, rice husk	α-Amylase	Baysal *et al.* (2003)
Penicillium sp.	Defatted soya bean cake	Protease	Germano *et al.* (2003)
C. fimbriata	Coffee pulp, coffee husk	Fruity aroma	Medeiros *et al.* (2003)
Lactobacillus amilophylus	Wheat bran	L (+)-Lactic acid	Naveena *et al.* (2005)
A. niger ATCC 16620	Tamarind seed powder, palm kernel cake	Tannase	Sabu *et al.* (2005)

Aspergillus niger	Sugarcane bagasse	Pectin lyase	Ramanujam *et al.* (2008)
Aspergillus niger GH1	Pomegranate Peel/Creosote Bush	Antioxidant Nutraceuticals	Aguilar *et al.* (2008)
Aspergillus niger	Pomegranate	Ellagic acid	Robledo *et al.* (2008)
Aspergillus niger	Desseded Sunflower Head	Pectinase	Patil *et al.* (2007)
Kluyveromyces marxianus	Sugar cane bagasse	Inulinase	Mazutti *et al.* (2007)
Aspergillus niger	Lignocellulosic substrates	Cellobiase	Rajoka *et al.* (2006)
Ceratocystis fimbriata	Coffee husks	Aroma compounds	Medeiros *et al.* (2006)
Rhizopus oligosporus	Pineapple waste	Phenolic antioxidants	Correia *et al.* (2004)
Aspergillus niger	Sucrose/Polyurethane foam	Invertase	Romero-Gómes *et al.* (2000)
Aspergillus japonicus	Agro-industrial residues	Fructooligosaccharides	Mussatto & Teixeira (2010)
Penicillium chrysogenum	Bagasse	Penicillin	Barrios-González *et al.* (1988)

Among these, filamentous fungi are the best studied for SSF, because, due to their hyphal growth, they can not only grow on the surface of the substrate particles, but also penetrate through them. During growth on such substrates, hydrolytic exo-enzymes are synthesized by the microorganisms and excreted outside the cells, helping in the access of carbon source and nutrients by the cells. This in turn promotes biosynthesis and microbial activities (Raimbault, 1998; Krishna, 2005).

2.2.3 **SUBSTRATE**

Substrate moisture and water activity play a very important role in SSF. Water relations in SSF must be critically evaluated. Water activity (a_w) of the substrate has a determinant influence on microbial activity. In general, the type of micro-organism that can grow in SSF systems is determined by a_w. The control of this parameter could be used to modify the metabolic production and product secretion of a microorganism. Moreover, the selection of the substrate is one of the most critical steps in solid substrate fermentation as it provides both nutrients and support. Nutritional requirement of the microorganism is a crucial parameter for its growth and sporulation. Sporulation is associated with the synthesis of macromolecules which are needed to produce the sporulation-specific structures and storage molecules needed for germination. The solid substrate, however, must contain enough moisture. Depending upon the nature of the substrate, the amount of water absorbed could be one or several times more its dry weight, which leads to a relatively high water activity (a_w) on the solid–gas interface, in order to allow a higher reaction rate of the biochemical process. Thus, the maintenance of an adequate moisture level in the solid matrix, along with suitable water activity, are essential elements for SSF processes (Krishna, 2005; Gervais and Molin, 2003; Marin-Cervantes *et al.*, 2008).

2.2.4 **BIOREACTORS**

Over the last decade, there has been a significant improvement in understanding of how to design, operate and scale up SSF bioreactors. The bioreactor is the heart of a fermentation process, wherein the raw material, under suitable conditions is converted to the desired product. SSF bioreactor systems are yet to reach a high degree of development, mainly due to the problems associated with solid beds like poor mixing and heat transfer characteristics and material handling. Many different bioreactors have been used in SSF processes and have been given different names by different authors. (Pandey, 2003). However, based on similarities in design and operation, SSF bioreactors can be divided into groups on the basis of how they are mixed and aerated.

Durand (2003) and Ruiz *et al.* (2007) reviewed different types of SSF fermenters, which were described and used for various purposes, incorporating several modifications for improved operation and performance. Among these several types of SSF reactors, rotating drum bioreactors provide relatively gentle and uniform mixing by improved baffle design, since there is no agitator within the substrate bed. These typically consist of a drum of cylindrical cross section lying horizontally. The drum is partially filled with a bed of substrate, and air is blown through the headspace. In rotating drums, the whole drum rotates around its central axis to mix the bed (Figure 2.3).

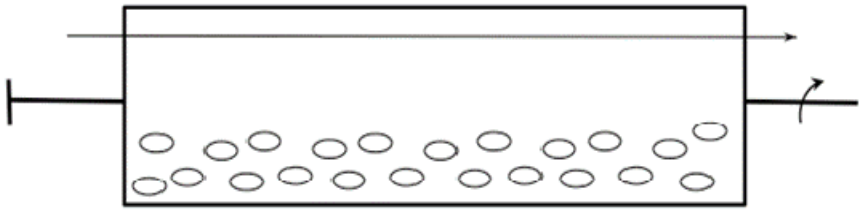

Figure 2.3. Rotating drum bioreactor

2.2.5 **SEAWEED**

Traditionally, seaweed cultivation is regarded as a kind of oceanic farming, because of its similarities to agriculture. The distribution of macroalgae is worldwide. They are abundant in coastal environments, primarily in coastal waters with suitable substrate for attachment. Seaweed also occurs as floating forms in the open ocean, and floating seaweeds are considered one of the most important components of natural materials on the sea surface. In the recent years, the seaweed cultivation has improved rapidly with the development of modern biotechniques. Moreover, seaweed has been an important component of food, feed and medicine in the Orient for several centuries. Seaweed have accounted for almost 3,000 natural products representing 20% of the chemistry reported from the marine realm. During the 1960s, the period when dedicated marine natural product laboratories were being established worldwide, more than 50% of newly reported natural products came from seaweed, though that number has steadily decreased and now hovers around 10% annually. In the Western world, seaweed is almost exclusively used for the production of important food hydrocolloids such as alginates, carrageenans, or agars. Moreover, seaweed contains important polysaccharides as sulfated fucose and laminarin and these polymers are located either in cell walls or within the cells serving as storage materials. (Radmer, 1996; Santelices, 1996; Blunt *et al.*, 2005; Vandendriessche *et al.*, 2006; Chaturvedi *et al.*, 2011).

According to FAO (2008), the harvest of wild stock accounted for about 1.1 million wet metric tons of annual world seaweed production in 2006, whereas 15.7 million metric tons were produced by aquaculture. For the all mentioned above, seaweed farming is already a significant industry, with a sophisticated technological basis, ranging from biotechnology to aquaculture, processing and marketing of the many products derived from these plants.

Seaweeds are classified into three broad groups based on their pigmentation: (1) brown seaweed (*Phaeophyceae*); (2) red seaweed (*Rhodophyceae*) and (3) green seaweed (*Chlorophyceae*) (McHugh, 2003). The structure of seaweed polysaccharides varies according to the species of seaweed. Seaweed contains a wide variety of acidic polysaccharides, some composed of uronic acids as the alginic acids and others made of

xylose, fucose, glucuronic acid and sulfate in different proportions (Dietrich *et al.*, 1995; Leite *et al.*, 1998).

The northern Portuguese coast is a biogeographic transition zone where many seaweed species have their distribution. Among these, the genus *Fucus* that comprises intertidal brown algae is composed by multiple species that are distributed within different but overlapping vertical limits all along northern Atlantic intertidal zone (Billard *et al.*, 2010; Coyer *et al.*, 2011; Zardi *et al.*, 2011). The seaweed *Fucus vesiculosus* is a member of the brown seaweed family (Figure 2.4).

Figure 2.4. *Fucus vesiculosus* from northern Portuguese coast

In general, the brown algae are known to have a high polysaccharides content and consist of three major types of components: (1) mineral or inorganic part, (2) alginates, (3) fucans and others carbohydrates (Figure 2.5). They have also proven to be rich sources of structurally diverse bioactive compounds with valuable pharmaceutical and biomedical potential (Shyamali *et al.*, 1988; Morris *et al.*, 1999; Merrifield *et al.*, 2004; Veena *et al.*, 2007). For this reason, the utilization of fucans as substrates in solid-state fermentation process provides an alternative avenue and value-addition on the production of different products as enzymes and bioactive compounds.

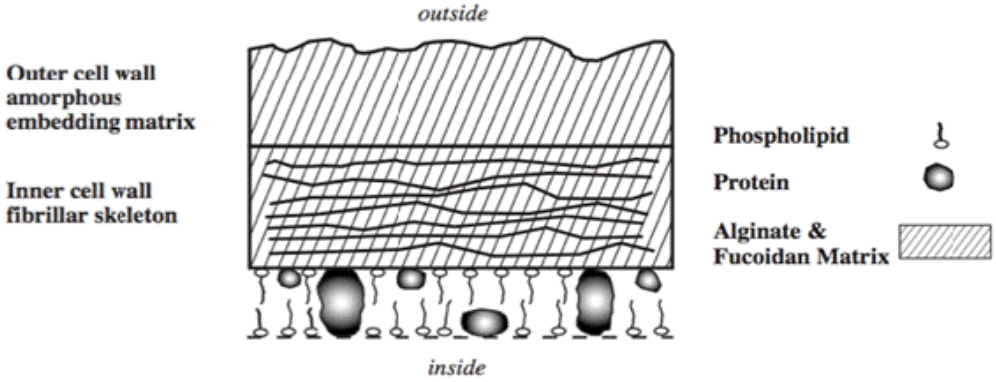

Figure 2.5**.** Cell wall structure in brown seaweed (Schiewer and Volesky, 2005).

2.2.6 **FUCOIDAN**

The active substance that is found in *Fucus vesiculosus* is fucoidan and is considered as a heteropolysaccharide (sulphated - polysaccharide) (Ale *et al.*, 2011). The fucoidan mainly derived from brown seaweed contains substantial percentages of L-fucose (44. 1 %), sulfate ester groups (26 %) and ash (31.1), also containing other monosaccharides (mannose, galactose, glucose, xylose) and uronic acids. However, its composition differs from source and season (Duarte, *et al.* 2001; Li *et al.*, 2008). Patankar *et al.* (1993), proposed the structure of fucoidan, consisting mainly of α-1-3-L-fucose. Figure 2.6, shows three structural motifs of fucoidan. These motifs do not depict the great diversity of fucoidan structures that are possible, but only shows a small range of common motifs.

In recent years, the fucoidan from seaweed has been used in a wide range of applications due to its potential biological activities. These include antitumor, immunomodulatory, antimutagenic, anticoagulant, antiviral, anti-inflammatory, hypolipidemic, renal and hepatic effects (Mayer *et al.*, 2004; Kang *et al.*, 2008). Some of these recent applications of fucoidans are shown in Table 2.2. Nevertheless, there are very few reports in the literature on the antioxidant capacity of fucoidan (sulphated polysaccharide). Rocha de Souza *et al.* (2007) obtained results that showed the beneficial effect of seaweed polysaccharides as antioxidant. Wang *et al.* (2009, 2010), reported that the fucoidan obtained from *L. japonica* possessed considerable antioxidant

activities using various established in vitro systems. In a recent work, Hou *et al.* (2012), reported the antioxidant activities of fucoidans from *L. japonica* with molecular weights between 1.0 – 144.5 kDa, respectively. The samples with molecular weights of 1.0, 3.8 and > 8.3 kDa have better hydroxyl radical scavenging activity.

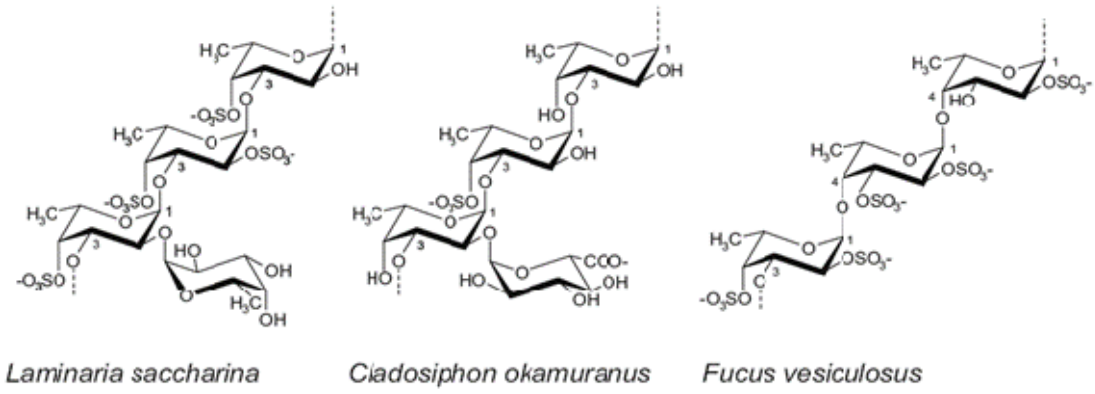

Figure 2.6**.** Structural motifs for fucoidans from three different brown seaweeds. (Cumashi *et al.*, 2007; Holtkamp *et al.*, 2009).

Table 2.2. Applications of fucoidan

Fucoidan source	**Application**	**Reference**
Fucus vesiculosus	Anticoagulant	Durig *et al.* (1997)
Fucus vesiculosus	Antiinflammatory	Park *et al.* (2012)
Undaria pinnatifida	Antitumor	Synytsya *et al.* (2010)
Undaria pinnatifida	Genotoxicity	Kim *et al.* (2010)
Fucus vesiculosus	Antithrombogenic/anticoagulant	Vesel *et al.* (2011)
Sargassum cymosum	Cytotoxicity	Lira *et al.* (2011)
Laminaria hyperborea	Intestinal microflora	Lynch *et al.* (2010)
Laminaria japonica	Renal damage	Wang *et al.* (2011)

On the other hand, the extraction methods for fucoidan vary in the literature. An important point to be taken in consideration on the extraction process is the presence of interfering substances (chemical components) (Chirinos *et al.*, 2007). For example, Rioux *et al.* (2007), extracted polysaccharides from brown seaweeds using selective solvents and Rodriguez-Jasso *et al.* (2011) reported the extraction of fucoidan using a microwave-assisted process. This technique consists in the penetration of microwave energy into the material structure, which produces a volumetrically distributed heat source due to molecular friction resulting from dipolar rotation of polar solvents and from the conductive migration of dissolved ions, accelerating the mass transfer of target compounds. In general, the compounds are extracted more selectively and quicker by this technique, with similar or better yields in comparison with conventional extraction processes (Rodriguez-Jasso *et al.*, 2011). In an excellent review, Ale *et al.* (2011), describe various methods of fucoidan extraction, for example, dilute acetic acid and hydrochloric acid extraction. Karmar *et al.* (2009) reported the water extraction of fucoidan from *P. tetrastromatica* brown seaweeds.

2.2.7 **FUCOIDANASE**

Enzyme production is a growing field of marine biotechnology, principally in the last 10 years (Holtkamp *et al.*, 2009). The global market for industrial enzymes is estimated as $3.3 billion in 2010. This market is expected to reach $4.4 billion by 2015 (BBC research, 2011).

Enzymes with known specificities that catalyze the degradation of fucoidan to produce low molecular weight fucoidans are an important tool for studying the structural peculiarities and biological role of this class of polysaccharides,.This special group of hydrolases, called fucoidanases (EC3.2.1.44) or α-L-fucosidases (EC 3.2.1.51) depend on its specificity for the glycosidic linkage (Giordano *et al.*, 2006). For the fucoidanases (or fucansulphatehydrolases), there are two different types known. One fucoidanase cleaves oligosaccharides from the end of the polysaccharide chain thus leading to lower molecular weight products. The other fucoidanase cleaves somewhere in the middle of the polysaccharide. An α-fucosidase only cleaves the α-L-fucosyl linkages at the non-reducing termini of the fucoidan. Fucoidanases are reported to be found principally in

marine organisms, and their activities are usually extremely low and could be extracted from hepatopancreas of invertebrates, marine bacteria marine fungi and, nowadays, terrestrial fungi (Yaphe and Morgan, 1959; Kitamura *et al.*, 1992; Bakunina *et al.*, 2000; Saki *et al.*, 2003; Urvantseva *et al.*, 2003; Tissot *et al.*, 2006; Rodríguez-Jasso *et al.*, 2008; El-Shahawi *et al.*, 2009; Rodríguez-Jasso et al, 2010; Moyra *et al.* 2012).

The enzymes can be both intra and extracellular. One of the difficulties in the purification of fucoidanase is the absence of simple methods for its identification and quantification.(Burtseva *et al.* 2000). Attia *et al.* (2010) reported a novel spectrofluorimetric method for measuring the activity of the α-L-fucosidase. This method was based upon measuring the quenching of the luminescence intensity of the produced yellow colored complex ion. Fucoidanase activity can be detected by a viscometric assay specific for endodepolymerases or by measurement of the increase in the content of reducing end groups (Kusaykin *et al.*, 2008). Table 2.3 gives an overview on fucoidan-degrading enzymes. The number of papers focused on fermentation conditions for fucoidanase production is small, making attractive the study of SSF as a potential alternative in the production of this type of enzymes.

Table 2.3. Fucoidan-degrading enzymes

Source	**Enzyme**	**Reference**
Charonia lampas	α-L-Fucosidase	Butters *et al.* (1991)
Sea sand	Fucoidanase	Furukawa *et al.* (1992)
33 species marine invertebrates	Fucoidan hydrolase	Burteseva *et al.* (2000)
Pectinidae	Fucoidanase	Kitamura *et al.* (1992)
Vibrio sp	Fucoidan hydrolase	Furukawa *et al.* (1992)
Littorinideae	α-L-Fucosidase	Kusaykin *et al.* (2006)
Strongylocentrotus	Fucoidan hydrolase	Sasaki *et al.* (1992)
Sphingomonaceae	Fucoidanase	Kim *et al.* (2008)

Kusaykin *et al.* (2006) studied the specificity of fucoidanases from. *Pseudoalteromonas citrea* KMM 3296 and the marine mollusk *Littorina kurila*. The maximum degree of cleavage of fucoidan was achieved by the fucoidanase from the marine bacterium *Pseudoalteromonas citrea* KMM 3296. Furukawa, *et al.*, (1992) purified three fucoidanases from *Vibrio* sp. N-5 by ammonium sulfate fractionation. These enzymes hydrolyzed gagome-fucoidan to give small oligosaccharides with sulfate as main product. Burtseva *et al.* (2000) analyzed thirty-three species of marine invertebrates from the Sea of Japan for its content of fucoidan hydrolases. It was found that fucoidan hydrolases of different specificities are fairly widely distributed among marine invertebrates. Kusaikin *et al.* (2004) reported that fucoidanases from the marine mollusk and marine bacterium have a similar specificity and hydrolyze predominantly the α-(1-3)-bond between the fucose residues in the polysaccharide molecule. Qian *et al.* (2003) reported the production of fucoidanase in solid-state fermentation using a marine fungus *Dendryphiella arenaria* TM94 obtaining a fucoidanase activity. In a recent work, Qianqian *et al.* (2011) reported the production of fucoidanase from *Fusarium sp.* (LD8) by SSF. Bakunina *et al.* (2002) reported an efficient degradation of fucoidan from brown seaweed at pH 6.5-7 resulting in the formation of sulfated α-L-fucooligosaccharides. The degradation activity remained at 40-50 ºC. Tanaka and Sorai, (1970) reported the almost complete hydrolysis of fucoidan with α-L-fucosidase from abalone liver (*Haliotis gigantean Gmelin*), that acted most likely as a exofucoidanase at pH = 5. Bilan *et al.* (2005) reported the hydrolytic activity of fucoidanase on the fucoidan from the brown alga *Fucus distichus* showing the formation of irregular regions of the native polysaccharides containing acetylated and partially sulfated repeating units.

2.3 CONCLUSIONS

Advances in the integration of bioprocess into industrial biotechnology offer potential opportunities for economic utilization of biomass marine such as seaweed. Moreover, the introduction of technologies as solid-state fermentation for the efficient use and conversion of fucoidan from seaweed is currently being promoted. Aditionally, the fucoidanases produced by SSF have a high potential use in the pharmaceutical and nutritional fields.

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CHAPTER 3

FUCOIDAN-DEGRADING FUNGAL STRAINS: SCREENING, MORPHOMETRIC EVALUATION AND INFLUENCE OF MEDIUM COMPOSITION

ABSTRACT

Ten different fungal strains from the genus *Aspergillus*, *Penicillum* and *Mucor* were screened for fucoidan hydrolyzing ability aiming to find microorganisms able to produce sulfated fucan-degrading enzymes. Screening was carried out by measuring the strains kinetic and morphometric behavior over plate assays using *Laminaria japonica* fucoidan as only carbon source, testing three nitrogen sources (urea, peptone and sodium nitrate). The selected fungal strains were subsequently used in submerged fermentations, which were performed for: 1) selection of the strains able to growth over fucoidan medium, and 2) media selection, testing the synergy of fucoidan with others sugars for inducing high enzyme titles. Radial expansion and hyphae parameters were observed for *Aspergillus niger* PSH, Mucor sp. 3P, and *Penicillium purpurogenum* GH2 grown only over fucoidan-urea medium. *A. niger* PSH showed the maximum enzymatic activity values, which were significantly different ($p<0.05$) from those achieved by the other selected fungi. Sucrose addition to fucoidan media proportioned the highest fucoidanase activity values for this fungal strain. This research allowed establishing optimal conditions for metabolites synthesis by fungal stains able to act toward fucoidan ramified matrix.

3.1 INTRODUCTION

Fucoidan is a polysaccharide widely found in brown algae cell walls. This compound has a wide variety of biological activities including anticoagulant, antithrombotic, antitumoral, and antiviral being the most relevant against hepatitis, herpes and human immunodeficiency (AIDS) viruses. The biological activity of fucoidans is determined by their specific chemical structure, which consist of a family of homo- and heteropolysaccharides composed mainly of fucose residues sulfated at positions 2 and/or 4 and bound by a-1,2- or a -1,3-O-glycosidic bonds. In addition to fucose, fucoidans may also contain mannose, xylose, galactose, and rhamnose sugars and uronic acids. Although the scientific and practical interest of studying the relation between structure and biology activity has been increased, a detailed elucidation of fucoidan chemical structure remains unknown (Alexeeva *et al.*, 2002; Ellouali *et al.*, 1993; McClure *et al.*, 1992).

Specific glycosyl hydrolases enzymes able to degrade fucoidan sulfated matrix are important tools to establish structural characteristics and biological functions of this polysaccharide. The literature available data mainly concern the fucoidanases of marine invertebrates such as *Haliotis* sp. and *Mizuhopecten yessoensis* (Sakai *et al.*, 2004; Urvantseva *et al.*, 2006), but the main reports of endo- and exo- fucoidanases have been found in marine bacteria as *Vibrio* sp., *Pseudoalteromonas citrea*, *Pseudomonas* sp., *Alteromonas* sp., *Flavobacteriaceae* sp., (Bakunina *et al.*, 2002; Furukawa *et al.*, 1992; Giordano *et al.*, 2006). Information on terrestrial and fungal microorganisms acting over this sulfated-polysaccharide is scarce. Therefore, the research for microorganisms with high fucoidan hydrolase activity remains a challenge.

Following the principle that polysaccharide degradation can be determined by monitoring microbial growth on plate media with a specific carbon source, polysaccharidases-producing microorganisms can be found from enrichment cultures on the target polysaccharide. Studies on image analyses of fungal strains have helped to relate morphological features to enzymes production aspects (pectinase and amylase), and the influence of culture media on growth patterns (Carlsen *et al.*, 1996; Minjares-Carranco *et al.*, 1997; Rodríguez-Gómez *et al.*, 2009). Fungal filamentous growth can

be interpreted on the basis of a regular cell cycle; therefore, mycelial growth and morphology can be mathematically described by validated kinetic models for the estimation of specific growth rate (μ) of molds on agar plates by image processing techniques (Larralde-Corona *et al.*, 1997; Loera and Viniegra, 1998).

The aim of this study was to identify fungal strains able to growth over fucoidan-based media and produce active fucoidanases. Specifically, the quantification of kinetic and morphology features over agar-plate aiming to establish the influence of media composition on growth patterns, was investigated. The interaction between different saline media and carbon sources as enzyme inducers in submerged fermentations was also evaluated.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 REAGENTS

Fucoidan of *Laminaria japonica* were purchased from Rizhao Jiejing Ocean Biotechnology Development Co., Ltd. Coomassie Plus (Bradford) Assay Kit were from Thermo Scientific Co. Anthrone reagent were from Prolabo, Normapur, Merck and 3,5-Dinitrosalicylic acid from Fluka, Chemika. Other reagents were all of analytical grade.

3.2.2 FUNGAL STRAINS AND INOCULUM PREPARATION

Ten filamentous fungi isolated from Northeast Mexican desert, which belong to the collection of the Food Research Department (DIA-UAC) from the University of Coahuila (Saltillo, Mexico), reported as microorganisms previously used for the induction of non constitutive enzymes (Cruz-Hernandez *et al.*, 2005), were used in the present work. The strains were identified in the Micoteca of the University of Minho (MUM, Braga, Portugal), being classified as five *Aspergillus* (*Aspergillus ustus* PSS,

Aspergillus niger ESH, *Aspergillus niger* GH1, *Aspergillus niger* PSH, and *Aspergillus niger* AA20), four *Penicillium* (*Penicillium pinophilum* EH2, *Penicillium pinophilum* EH3, *Penicillium* sp. ESS, and *Penicillium purpurogenum* GH2) and one *Mucor* sp. (3P). For inoculum preparation, fungal spores were transferred to potato dextrose agar medium, and incubated at 30 °C for 7 days. Spores were scraped into 0.01% (v/v) Tween 80 solution and counted in a Neubauer chamber to be inoculated in the fermentation media.

3.2.3 CULTURE MEDIA AND FERMENTATION CONDITIONS

3.2.3.1 Screening of Fungal Strains

Different culture media containing fucoidan as sole carbon source were used for the fungal strains screening. Culture media MM were composed by (g L^{-1}): fucoidan (5.0), nitrogen source (2.0), and agarose (10.0), dissolved in 100 mM acetate buffer (pH 4.5). Urea, peptone and sodium nitrate were tested as nitrogen sources. For comparative experiments, culture media CZ, containing Czapek dox mineral salts, were used. Such media were composed by same carbon and nitrogen sources of media MM plus the addition of mineral salts in the following concentrations (g L^{-1}): KH_2PO_4 (1.0), MgSO4 (0.5), and KCl (0.5). Culture media containing only nitrogen sources were used as growth blank.

After preparation, the media were poured on 60 mm Petri plates and inoculated in the center with a spore suspension containing $1x10^6$ conidia mL^{-1}. The plates were statically incubated at 30 ºC during 5 days. Cultivations were done in triplicate to each fungal strain in each different culture media.

3.2.3.2 Fermentation Conditions for Microorganism Selection

In this stage, the fermentation assays were carried out in 250 mL Erlenmeyer flasks containing 50 mL of culture media MM composed by fucoidan and urea, 10.0 and 5.0 g L^{-1}, respectively. Culture media had their pH adjusted to 5 by addition of HCl 0.5M

solution and were sterilized by microfiltration (membrane filters of 0.2 µm, Millipore). The flasks were inoculated with $1x10^6$ spores mL^{-1}, and maintained at 30 °C, 140 rpm, during 5 days. Cultivations were done in duplicate to each fungal strain selected from the previous stage.

3.2.3.3 Fermentation Conditions for Media Selection

These assays were performed for evaluation of the interaction effect between fucoidan (primary carbon source) and a secondary carbon source: glucose, sucrose, lactose, fructose, or sodium acetate. The fermentation media were composed by (g L^{-1}): fucoidan (10.0), secondary carbon source (5.0), and urea as nitrogen source (5.0), supplemented with Czapek Dox (CZ) or Pontecorvo (PC) mineral salts. For CZ media, the mineral salts and their respective concentrations were the same described in the section 2.3.1. For PC media, the mineral salts and their concentrations were (g L^{-1}): KH_2PO_4 (1.5), $MgSO_4$ (0.5), KCl (0.5), $FeSO_4 \times 7H_2O$ (0.001), $ZnSO_4 \times 7H_2O$ (0.001), $MnCl_2 \times 4H_2O$ (0.001), and $CuSO4 \times 5H_2O$ (0.001).

The fermentations assays were carried out in 250-mL Erlenmeyer flasks containing 50 mL of culture media (pH adjusted to 5 by addition of HCl 0.5 M) sterilized by microfiltration (membrane filters of 0.2 µm, Millipore). The flasks were inoculated with $1x10^6$ spores mL^{-1}, and maintained at 30 °C, 140 rpm, during 5 days. Cultivations were done in duplicate only for the previous selected fungal strain.

3.2.4 ANALYTICAL METHODS

3.2.4.1 Sugars Consumption

Culture media consumption was quantified as the intake percentage of total sugars (TS), which were quantified by Anthrone method (Dreywood, 1996) using glucose as standard.

3.2.4.2 Biomass and Radial Growth Rate

Fungal biomass during the submerged fermentations was evaluated by dry weight, where the mycelium obtained after filtration (membrane filter 0.45 μm, 47 mm) was dried at 150 W for 20 min.

Radial growth rate (U_r) was kinetically monitored measuring colony diameters every 24 h for 5 days, using a digital micrometer (Mitutoyo 293-561, Japan). Slopes of radial growth were estimated by linear regression (R^2=0.95) and expressed as μm h^{-1}. Final colonies were saved for being observed on the microscope in order to obtain average data for hyphal length and diameter to each fungal strain. Specific growth rates (μ, h^{-1}) were calculated according to the following model (Loera and Viniegra, 1998):

$$\mu = \frac{U_r \ \ln(2)}{L_{av} \ \ln\left(\frac{L_{av}}{D_h}\right)} \qquad (1)$$

where U_r was the radial growth rate (μm, h^{-1}), L_{av} was the average length of leading hyphae in the periphery of the colonies (μm), and D_h was the mean diameter of hyphal tubules (μm).

3.2.4.3 Protein and Enzymatic Activity

Total protein concentration in fungal extracts was determined with a Bradford protein assay kit (Bradford reagent, Pierce). Enzymatic activity was measured by the dinitrosalicylic acid technique, which estimate the reducing sugars released during the reaction between 900 μL of substrate (fucoidan 10 g L^{-1} in 200 mM acetate buffer, pH 4.5) and 100 μL of enzyme extract, at 37 ºC for 24 h. One unity (U) of enzyme was defined as the amount of enzyme able to release 1 μmol of reducing sugars per h. The data correspond to triplicates of independent experiments.

3.2.5 IMAGE ACQUISITION

For image analyses measurements, a digital camera AxioCam HRc (Zeiss, Oberkochen) mounted on a Zeiss Axioskop microscope (Zeiss, Oberkochen) was used. Phase contrast with 10X and 40X objective lenses were selected for hyphal length (L_{av}) and diameter (D_h) measurements, respectively. Three plates of each fungus in each different medium were analyzed. The observation fields were selected randomLy along the entire periphery plate, and samples of 30 hyphal tubes per plate were quantified. The obtained images were analyzed in an Axiovision Software version 4.7. Mean values were calculated by a frequency distribution.

3.2.6 CALCULATION OF METABOLIC PARAMETERS

The metabolic parameters including specific growth rate (μ), biomass yield ($Y_{X/S}$), enzyme yield ($Y_{E/X}$), and enzyme productivity (P_R) were calculated. Specific growth rate was obtained from growth curves fitted by Microsoft Excel's Solver tool, using the logistic equation. The biomass yield was defined as the amount of biomass produced per gram of sugar consumed; the enzyme yield, as the enzyme activity per gram of biomass; and the enzyme productivity, as the rate of enzyme synthesis per liter per hour.

3.2.7 STATISTICAL ANALYSES

The difference among samples was verified by using the Tukey's range test. A *p* value of less then 0.05 was regarded as significantly different. Statgraphics Plus for Windows version 4.1 was the software used for data analysis.

3.3 RESULTS & DISCUSSION

3.3.1 STRAINS SELECTION ON PETRI PLATES

Growth fungal evaluation over fucoidan-based media was carried out with the purpose of find terrestrial microorganisms able to degrade this complex polysaccharide. From the ten strains assessed in the three different nitrogen sources, only *Aspergillus niger* PSH, *Penicillium purpurogenum* GH2, and *Mucor sp*., showed mycelium presence over fucoidan-urea plates in MM medium after 24 h of inoculation. Among these three selected strains, *Mucor sp.* 3P and *A. niger* PSH colonies completely covered the agar plate after 6 days of incubation while *P. purpurogenum* GH2 required more than 10 days of cultivation to invade half of Petri plate (Figure 3.1).

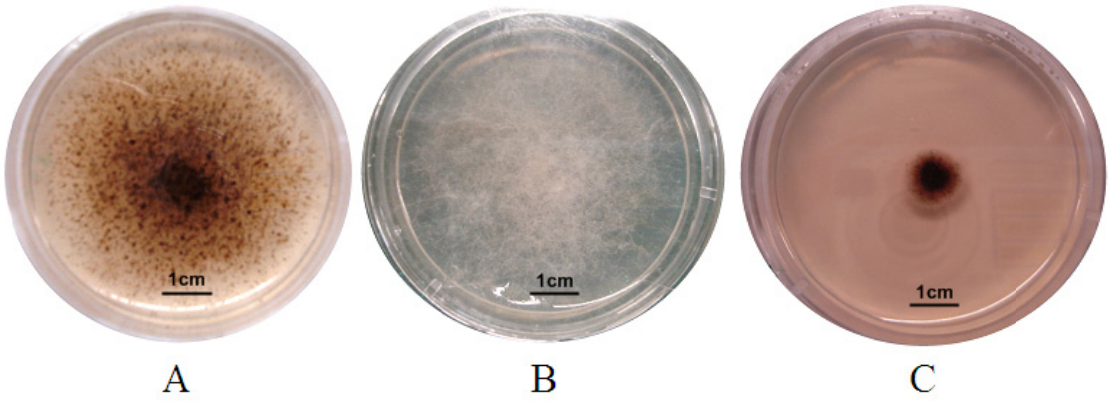

Figure 3.1. Microorganism with growth presence over fucoidan-urea dishes: A) *Aspergillus niger* PSH*; B) Mucor sp.* 3P; C) *Penicillium purpurogenum* GH2

On the contrary of urea medium, the culture media containing other nitrogen sources (peptone or sodium nitrate) did not show any strain growth. Fungal colonies formation over urea could have been influenced by the induction of urease, which could have promoted the extracellular fucoidanase production. The urease enzyme is usually constituve in fungal mycelia, and is able to degrade urea to ammonium and CO_2. Ammonium ions are readily translocated and can be assimilated into glutamate and

glutamine, which serve as precursors for the biosynthesis of many important cellular macromolecules (Kavanagh, 2005; Moure, 1998). In addition, urea appears to be the major nitrogen source in the sea, generally present at levels of 0.1-1 μM (Antia *et al.*, 1991; Collier *et al.*, 1999); therefore, it can be a natural promoter of the fucoidan hydrolytic enzyme.

3.3.2 HYPHAL GROWTH MEASUREMENTS

The image analyses measurements of hyphal length and diameter (Figure 3.2) were useful to classify fungal strains tested in selective culture media. Table 3.1 shows the morphological parameters obtained by fungal cultivation on fucoidan-urea Petri plates, comparing MM and CZ media. Differences among the fungal strains and media were perceptible; but, all the strains showed a direct relation between the kinetic (U_r) and micrometrical parameters (L_{av} and D_h).

For each evaluated medium, the highest parameter values were always obtained by cultivation of the *Mucor* 3P strain, while *P. purpurogenum* GH2 gave the lowest results. Microscopic observation of hyphal diameters (D_h) did not show significant differences among the fungal strains grown on minimal medium (MM). On the other hand, the hyphal length (L_{av}) of *Mucor* 3P had an average value 36% higher than that measured for *A. niger* PSH; which can be expressed as a major degree of branching. Mineral salts addition to the media (CZ) enhanced germination and growth of germ tube, allowing higher conidial elongation (L_{av}). On average, when cultivated in CZ media, *Mucor* 3P, *A. niger* PSH, and *P. purpurogenum* GH2 presented elongation rates 33%, 12% and 29% higher than those obtained in MM media; however, hyphal diameter was only significantly influenced on *Mucor* 3P. A possible explanation for this is that length and diameter are not regulated at the same physiological level during the growth process, since the hyphae diameter is defined at the beginning of the process, while the hyphae length remains elongating for a longer time. In addition, it was also found that elongation and diameter of the hyphae are closely related to the size of the spores, varying thus for the different fungal species (Juniper and Abbott, 2006). Therefore, the large spores of *Mucor sp*. provided fast growing of thick hyphae, whereas the small spores of *Aspergillus* and *Penicillium* species produced slow growing of thin hyphae.

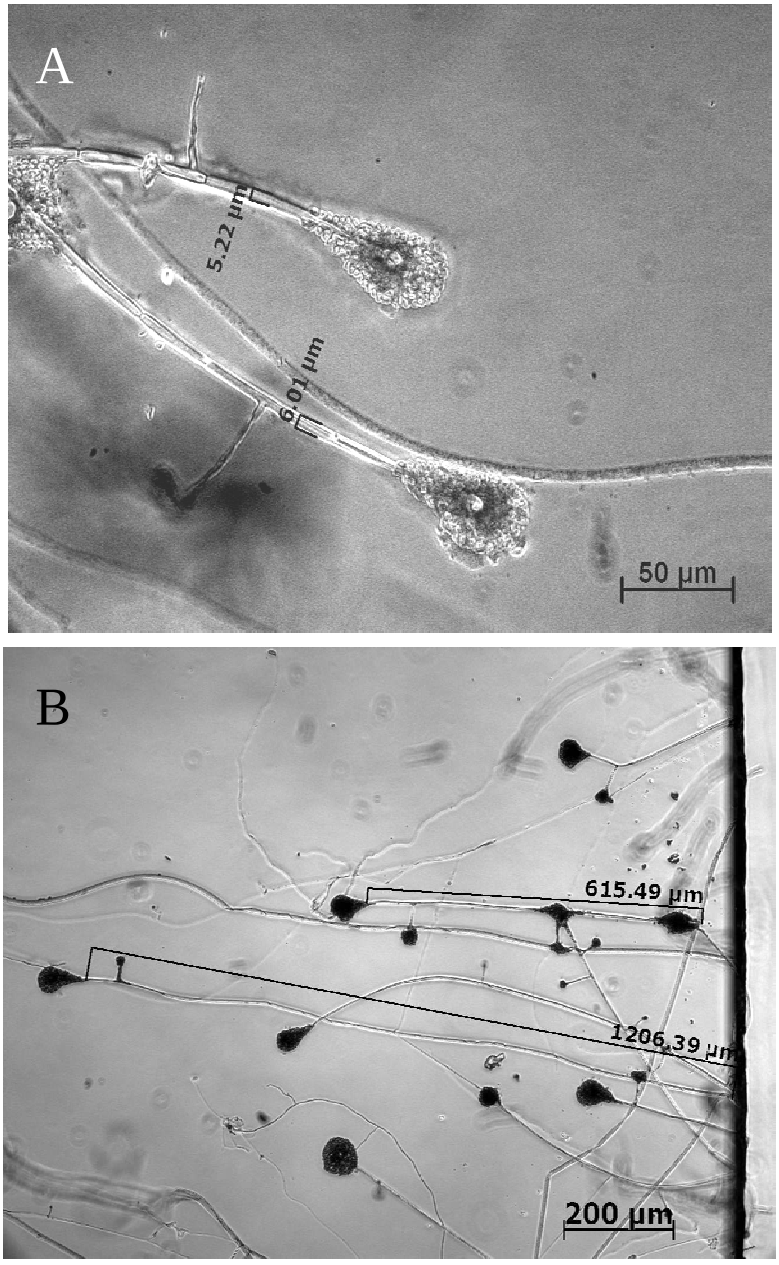

Figure 3.2**.** Image analysis measurement on contrast phase photography of *Mucor sp.* 3P growth on fucoidan-urea minimal medium (MM). (A) hyphae diameter, magnification 40X, and (B) hyphae length, magnification 10X

Table 3.1. Growth parameters of fungal strains cultivated on fucoidan-urea Petri plates.

Cultivation Media	Strain	U_r (µm h^{-1})	L_{av} (µm)	D_h (µm)	μ (h^{-1})
Minimal (MM)	*Mucor* 3P	579.90 ± 0.01	251.63 ± 89.21	4.44 ± 0.89	0.40
	A. niger PSH	350.43 ± 0.03	184.47 ± 48.18	4.41 ± 0.77	0.37
	P. purpurogenum GH2	136.93 ± 0.09	158.99 ± 66.07	3.40 ± 1.17	0.16
Czapek (CZ)	*Mucor* 3P	755.07 ± 0.01	336.68 ± 107.69	7.05 ± 1.72	0.40
	A. niger PSH	390.67 ± 0.01	208.33 ± 61.77	5.37 ± 1.06	0.36
	P. purpurogenum GH2	232.80 ± 0.16	206.29 ± 53.09	3.51 ± 0.80	0.19

U_r: Radial growth rate; maximal rate of extension of leading hyphae

L_{av}: Average length of hyphae in the periphery of the colonies

D_h: Mean diameter of hyphal tubules

µ: Specific growth rate

Among the three evaluated fungal strains, *Mucor sp.* 3P gave the highest specific growth rate (μ) values (Table 3.1). Similar results were obtained by cultivation of *A. niger* PSH in both media (MM and CZ), while *P. purgurogenum* GH2 provided the lowest results. Besides the highest specific growth rate, *Mucor* 3P gave also the highest maximal rate of extension of leading hyphal (U_r, μm h^{-1}), mainly when CZ medium was used.

The specific growth rate (μ, h^{-1}) is a valid physiological parameter for comparing fungal growth patterns measuring mycelial branching frequencies in order to estimate biomass formation. Cumulative percentage profiles of the total measured hyphae in CZ and MM media were then plotted against the hyphae length and diameter (Figure 3.3). High polydispersity was observed for the three fungi, as an evidence of the heterogeneous hyphae tips, which can be a response of metabolic stress at the induced media composition. No differences on diameter profile in *A. niger* PSH and *Mucor* 3P in MM media were observed (Figure 3.3B), and thus, medium assimilation was only differed by the length profile (Figure 3.3A). The presence of fungal colonies evidence that such microorganisms are able to excrete metabolites with hydrolytic action over the fucoidan branched structure.

3.3.3 PRODUCTION OF FUCOIDAN DEGRADING ENZYMES BY SELECTED STRAINS

Submerged fermentation experiments were carried out for the strains that showed capacity to grow over fucoidan plates (*A. niger* PSH, *P. purpurogenum* GH2 and *Mucor sp.* 3P). During their cultivation under submerged fermentation conditions they presented dispersed mycelial filaments, a particular morphological form that can be resulted from the nature of the inoculum as well as the minimal medium composition used in the experiments (Papagianni, 2004).

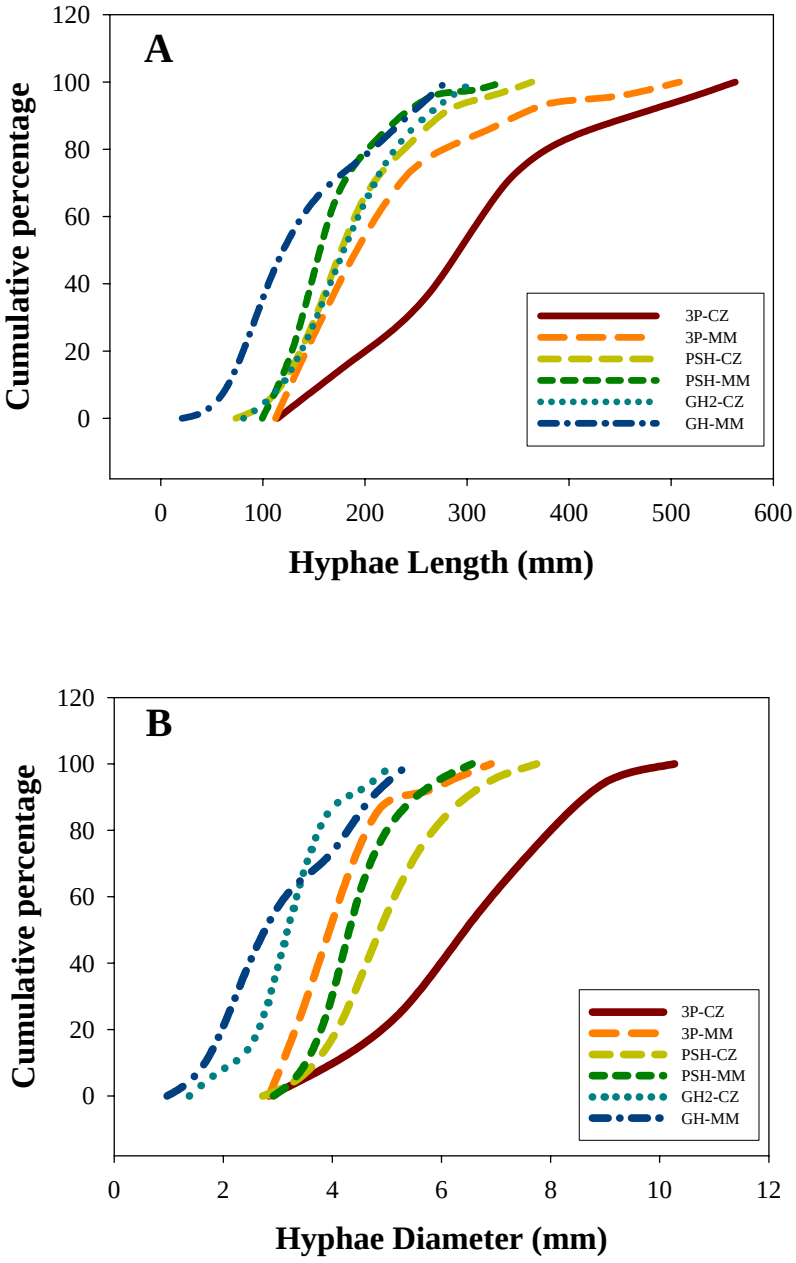

Figure 3.3. Cumulative percentage profile of hyphae length (A) and diameter (B) during the fungi cultivation in minimal (MM) and czapek (CZ) media. 3P: *Mucor sp.*; PSH: *Aspergillus niger*; GH2: *Penicillium purpurogenum*

Concerning to biomass production, the highest results were obtained with *Mucor sp.* 3P and *A. niger* PSH (0.849 and 0.464 g L^{-1}, respectively) after 48 h cultivation, time after which both strains reached the stationary phase (Figure 3.4A). On the contrary, as previously observed on agar plates experiments, *P. purpurogenum* GH2 showed the lowest biomass growth, reaching values closed to 0.274 g L^{-1}. Statistical analysis of these data (Table 3.2) only revealed differences significant at 95% confidence level between *Mucor sp.* 3P and *P. purpurogenum* GH2. The maximum substrate consumption obtained with *Mucor sp.* 3P was also statistically different of those obtained with the other fungal strains. By contrast, the enzyme activity was highest for *A. niger* PSH, with values oscillating from 0.441 to 0.613 U L^{-1} after 48 h of fermentation (Figure 3.4B), which were significantly different (p<0.05) of those obtained for *P. purpurogenum* GH2 and *Mucor sp.* 3P. In brief, *Mucor sp.* 3P was the strain with highest ability to growth and consume substrate; otherwise, *A. niger* PSH showed the highest enzymatic activity and its biomass production was not statistically different from the results achieved by *Mucor sp.* 3P. Based on these results, *A. niger* PSH was the fungal strain selected for use in the subsequent step.

Table 3.2. Multiple comparison procedure to discriminate among the biomass, substrate consumption and enzymatic activity means obtained during the submerged fermentation for microorganism selection.

Strain	Biomass (g L^{-1})	Substrate (g/100g TS)	Enzyme activity (U L^{-1})$^{\alpha}$
Mucor sp. 3P	0.85 ^{b}	17.17 ^{b}	0.17 ^{a}
A. niger PSH	0.46 ^{ab}	9.76 ^{a}	0.61 ^{b}
P. purpurogenum GH2	0.27 ^{a}	11.88 ^{a}	0.22 ^{a}

$^{\alpha}$ U L^{-1}= (μmol h^{-1}) L^{-1}

Different letter means values statistically different at 95% confidence level.

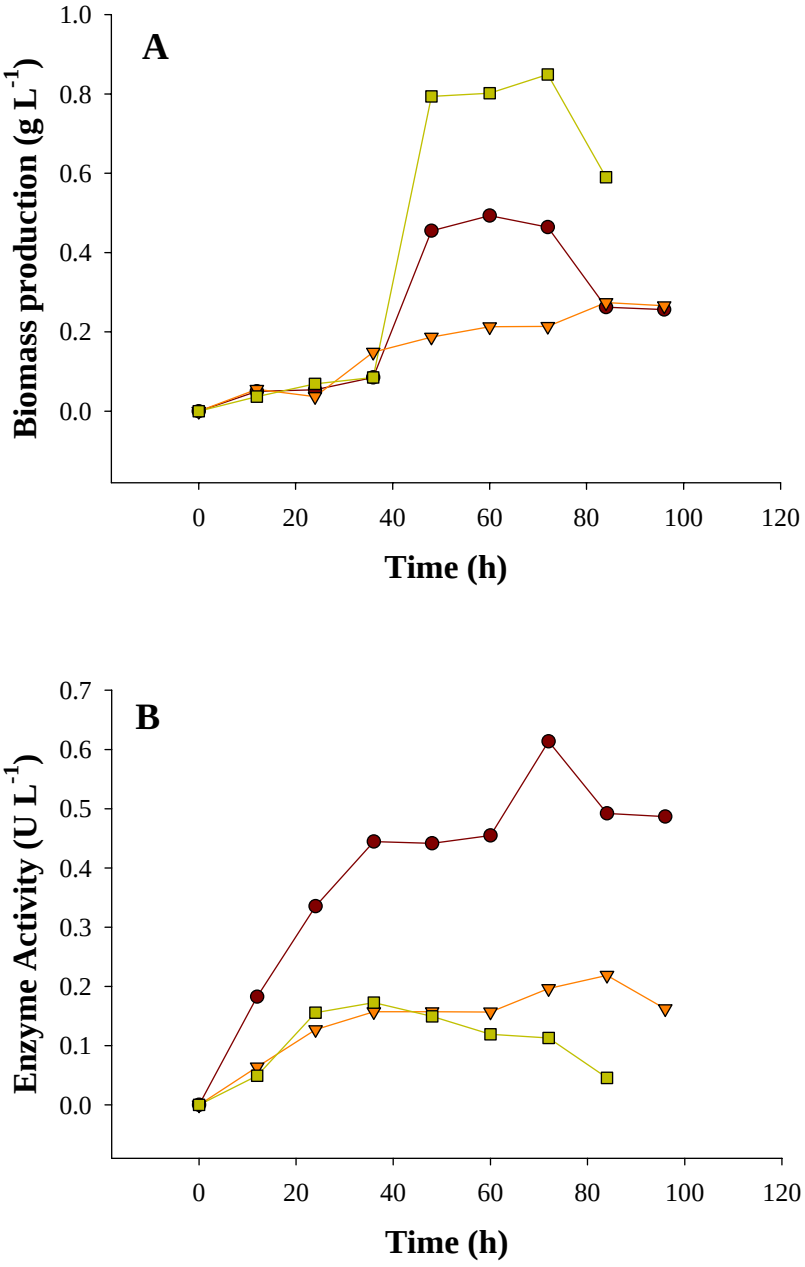

Figure 3.4. Submerged fermentation over fucoidan-urea medium of *Aspergillus niger* PSH (●), *Mucor sp.*, (■) and *Penicillium purpurogenum* GH2 (▼).
A) Biomass production; B) Enzyme activity

3.3.4 EFFECT OF COMBINED MEDIA ON BIOMASS GROWTH, SUBSTRATE CONSUMPTION, AND INDUCTION OF FUCOIDAN DEGRADING ENZYMES

The biomass results obtained during the *A. niger* PSH cultivation in the combined media are shown in Figure 3.5. Note that the maximum biomass production (5.79 g L^{-1}) was reached on Czapek medium containing fucoidan supplemented with sucrose, but the strain also grew well on the culture media supplemented with glucose or fructose (3.83 and 3.10 g L^{-1}, respectively). On the contrary, low mycelia production was observed when using fucoidan as sole carbon source or supplemented with lactose or sodium acetate as secondary carbon source, being not observed significant differences (p<0.05) among these three assays (Table 3.3).

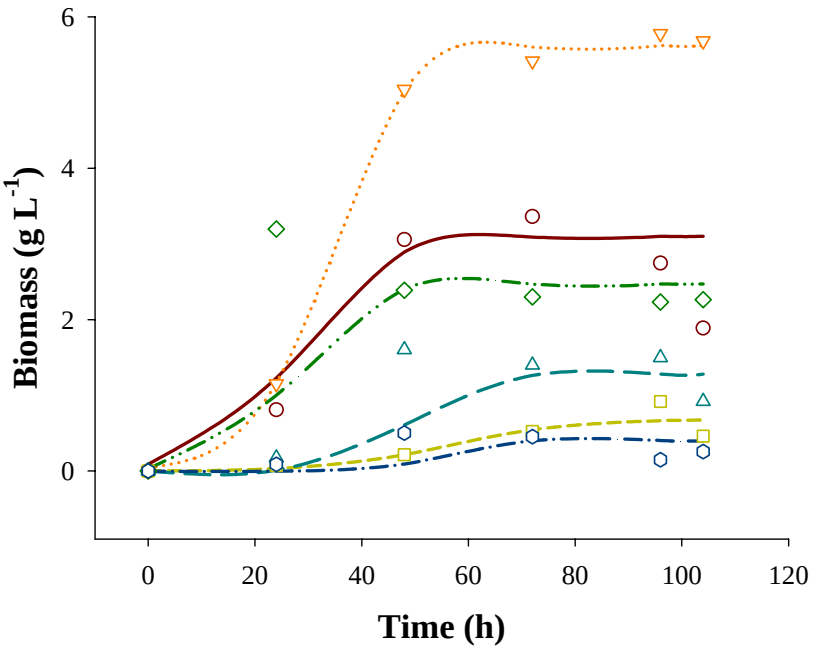

Figure 3.5. Biomass production of *Aspergillus niger* PSH during the submerged fermentation in Czapek medium containing fucoidan supplemented with: Glucose (●,—); Sucrose (▼,····); Lactose (■,---); Fructose (◆, -·-·-), or Sodium acetate (▲, – –). Medium containing Fucoidan as sole carbon source (●, -·-·-). Symbols are experimental data; Lines are calculated data adjusted by logistic equation (R^2 > 0.90)

Table 3.3. Multiple comparison procedure to discriminate among the biomass, substrate consumption, enzymatic activity, and protein means obtained during the submerged fermentation for media selection.

Culture media[α]	Biomass ($g\ L^{-1}$)	Substrate (g/100 g TS)[β]	Enzyme activity ($U\ L^{-1}$)[δ]	Protein ($mg\ L^{-1}$)
Glu/Fuc	3.84 [b]	79.93 [d]	0.67 [b]	15.03 [b]
Suc/Fuc	5.80 [c]	44.28 [c]	2.77 [e]	22.81 [c]
Fru/Fuc	3.11 [b]	53.38 [c]	0.00 [a]	16.00 [bc]
Lac/Fuc	0.92 [a]	28.84 [b]	0.93 [c]	4.83 [a]
Ac/Fuc	1.62 [a]	20.03 [a]	0.20 [a]	3.84 [a]
Fuc	0.53 [a]	8.52 [a]	1.88 [d]	4.81 [a]

[α] Glu: Glucose; Suc: Sucrose; Fru: Fructose; Lac: Lactose; Ac: Sodium acetate; Fuc: Fucoidan

[β] TS: total sugars

[δ] $U\ L^{-1}$ = (μmol /h)/L

Different letter means values statistically different at 95% confidence level.

Regarding the substrate consumption, the best results, once more were obtained in media supplemented with sucrose, glucose or fructose (Table 3.3). However, in this case the highest intake (79.93% of total sugars) was obtained in glucose supplemented medium. Sodium acetate addition did not promote significant differences from the medium containing fucoidan, but the addition of lactose favored the sugars consumption compared with this medium, although such consumption did not reflect in a highest biomass production. Otherwise, sucrose supplemented medium showed lower substrate consumption than that obtained in glucose supplemented medium, which means that sucrose supplementation allowed a higher cellular growth per substrate consumed, as can be confirmed by the $Y_{X/S}$ values given in Table 3.4.

Fucoidan hydrolytic enzymes were only expressed as extracellular metabolites in all the evaluated Czapek media supplemented with a secondary carbon source, being not detected any intracellular activity for all the studied media. Enzyme activity was highest in the sucrose supplemented medium, whose value (2.77 $U\ L^{-1}$) was significantly

different (p<0.05) of that attained in the medium containing fucoidan as sole carbon source (1.88 U L^{-1}) (Table 3.3).

Table 3.4. Kinetic parameters obtained during the fungal fucoidanase production by submerged fermentation using different culture media.[α]

Culture media[β]	$Y_{X/S}$	$Y_{E/X}$	P_R
Glu/Fuc	0.32	0.17	0.09
Suc/Fuc	0.87	0.48	0.40
Fru/Fuc	0.39	0.00	0.00
Lac/Fuc	0.21	1.02	0.08
Ac/Fuc	0.54	0.13	0.04
Fuc	0.62	3.55	0.50

[α] $Y_{X/S}$= g biomass per g substrate; $Y_{E/X}$ = U per g biomass; P_R = U L^{-1} h^{-1}

[β] Glu: Glucose; Suc: Sucrose; Fru: Fructose; Lac: Lactose; Ac: Sodium acetate; Fuc: Fucoidan

Kinetic profiles of fucoidanase activity are shown in Figure 3.6, which reveals a constant behavior between 48-96 h and 72-96 h of fermentation for sucrose-supplemented and fucoidan media, respectively; however, after 96 h the activities expression decreased abruptly in both media. Curiously, only any fucoidanase activity was detected in fructose supplemented medium, suggesting that the good results of biomass production and substrate consumption observed above could be related to the production of other proteins. As can be seen in Table 3.3, total protein production was highest in media supplemented with sucrose or fructose (22.80 and 16.03 mg L^{-1}, respectively), while the lowest protein values were found for the medium supplemented with sodium acetate, which did not show significant differences from the medium containing only fucoidan.

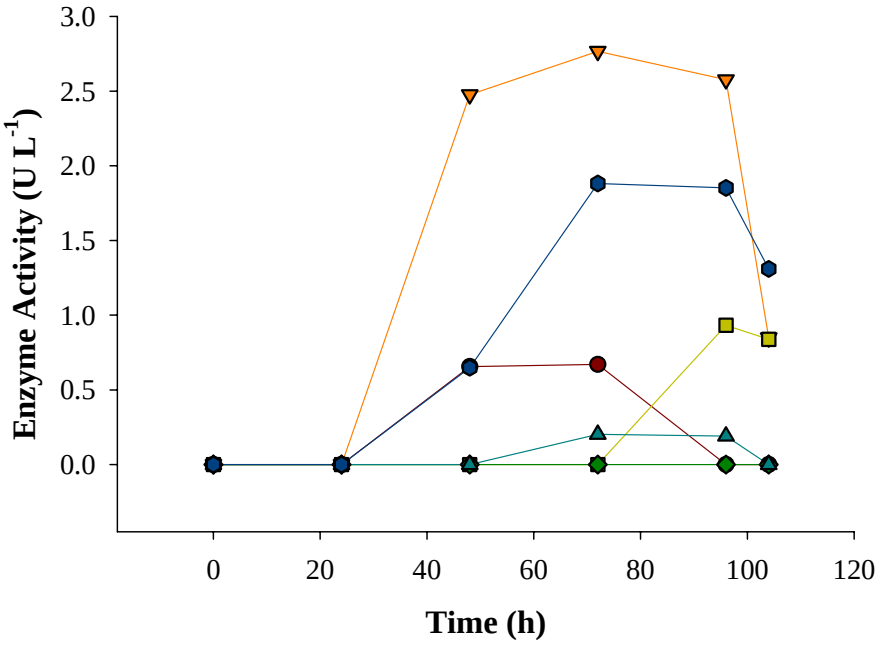

Figure 3.6. Fucoidanase activity of *Aspergillus niger* PSH during the submerged fermentation in Czapek medium containing fucoidan supplemented with: Glucose (●); Sucrose (▼); Lactose (■); Fructose (◆), or Sodium acetate (▲). Medium containing Fucoidan as sole carbon source (●)

The kinetic parameters obtained during the fungal fucoidanase production by liquid fermentation using different culture media are shown in Table 3.4. Confirming the above discussed idea, the highest biomass yield ($Y_{X/S}$ = 0.87 g g^{-1}) was obtained in Czapek medium containing fucoidan supplemented with sucrose. On the other hand, the highest enzyme yields ($Y_{E/X}$ = 3.55 and 1.01 U g^{-1}) were achieved in the media containing fucoidan as sole carbon source or supplemented with lactose, which can be explained by the low biomass formation attained in these media. Enzyme productivity values (P_R) showed that sucrose supplemented medium allowed the synthesis of fucoidan degrading enzymes in a rate similar to that obtained in the medium containing only fucoidan as carbon source.

It is worth mentioning that when Pontercorvo saline medium was used instead of Czapek, all the tested carbon sources also enabled the biomass formation; and likewise on Czapek medium, sucrose supplementation gave the highest values (4.77 g L^{-1}) followed by fructose and glucose supplementations (3.46 and 3.31 g L^{-1}, respectively). Lactose and sodium acetate supplemented media did not show significant differences from the lowest values obtained with fucoidan as sole carbon source. However, fucoidanase enzymatic activities were not detected in theses assays, suggesting that the synthesis of this enzyme could have been inhibited by the presence of microelements (Ca, Mg, Mn, and Cu) in the Pontecorvo formulation.

As a whole, the results obtained in the present work can be well compared with those reported for marine bacteria and invertebrates from other sources (Alexeeva *et al.*, 2002; Urvantseva *et al.*, 2006; Burtseva *et al.*, 2000). The differences of enzyme activity synthesis observed for the six evaluated media are probably due to the specificity of the enzymes production for some features of the substrate structure, combining simple sugar content and the complex fucoidan polysaccharide, with structural impediment such as the different position of sulfate groups and their sulfatation degree.

3.4 CONCLUSIONS

In conclusion, the use of agar plate quantitative method was an important tool for the establishment of preliminary physiological differences in fungal strains performance as enzymes producers. *Aspergillus niger* PSH, *Penicillium purpurogenum* GH2 and *Mucor sp.* 3P are able to growth on different fucoidan-urea media; however, only *A. niger* showed great importance for the synthesis of sulfated fucan-degrading enzymes. Differences in the culture medium composition had a significant effect on the fungi growth, and the production of secondary metabolites was substantially affected. Sucrose supplemented medium proportioned the best results, while the sodium acetate addition did not show any significant improvement (p<0.05) in the results obtained from fucoidan as sole carbon source. These are the first results describing the production of enzymes from terrestrial fungus with ability to degrade fucoidan.

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CHAPTER 4

MICROWAVE-ASSISTED EXTRACTION OF SULFATED POLYSACCHARIDES (FUCOIDAN) FROM BROWN SEAWEED

ABSTRACT

Sulfated polysaccharides (fucoidan) were recovered from brown seaweed *Fucus vesiculosus* by microwave-assisted extraction (MAE). Different conditions of pressure (30 to 120 psi), extraction time (1 to 31 min), and alga/water ratio (1/25 to 5/25 g mL^{-1}) were evaluated during this process aiming to establish a condition to maximize the extraction results. The alga degradation (%), total sugar yield (%), and SO3 content (%) were also determined to each experimental condition. All the studied variables presented significant ($p<0.05$) influence on fucoidan yield. MAE at 120 psi, 1 min, using 1 g alga/25 mL water was the best condition for the fucoidan recovery. L-fucose was the main constituent of this polysaccharide, which also contained xylose and galactose. MAE under optimum reaction conditions was an effective method to recover fucoidan from *Fucus vesiculosus*. This method required short extraction times, and non corrosive solvents, resulting in reduced costs and being an environmentally friend technique.

4.1 INTRODUCTION

Marine algae, or seaweeds, contain several physiologically bioactive compounds with important economical relevance, such as polysaccharides and iodine organic products, macro and micro elements, vitamins and unsaturated fatty acids (Bhakuni and Rawat, 2005; Craigie, 2010). Brown seaweeds are the second most abundant group of marine algae comprising about 2,000 species. Among them, *Ascophyllum spp*., *Fucus spp*., *Laminaria spp*., *Sargassum spp*., and *Turbinaria spp*. are the most commonly used on industrial level (Hong *et al.*, 2007). Recent studies have demonstrated that brown algae contain biologically active substances that can be used as anticoagulant, antithrombotic, anti-inflammatory, anti-tumor, contraceptive, and anti-viral, for the treatment of several diseases (Synytsya *et al.*, 2010; Wang *et al.*, 2010a). Such properties have been attributed to the sulfated polysaccharides fucoidans in the algae cell wall structure (Ellouali *et al.*, 1993; Berteau and Mulloy, 2003; Queiroz *et al.*, 2008).

Fucoidans may constitute up to 25–30% of the alga dry weight, depending on the seaweed specie and, to a lesser extent, on season. These polysaccharides are composed by α–1,3-backbones or repeating disaccharide units of α–1,3- and α–1,4-linked fucose residues with branchings attached at C2 positions. Depending on the structure of the main chain, fucoidans may be sulfated at C4, C2 or in both positions of the fucose units. Besides fucose, fucoidans may also contain mannose, xylose, galactose, and rhamnose sugars, and uronic acids (Kusaykin *et al.*, 2008; Rodríguez-Jasso *et al.*, 2010).

Sulfated polysaccharides are generally extractable with hot water, dilute acid, or dilute alkali, by using large solvents volume and long extraction times (Marais and Joseleau, 2001; Rioux *et al.*, 2007; Wang *et al.*, 2008; Yang *et al.*, 2008). In the last decade, microwave-assisted extraction (MAE) has been successfully applied for extraction of numerous biologically active compounds from a wide variety of natural resources (Sosa-Ferrera *et al.*, 2005; Périno-Issartier, 2010; Wang *et al.*, 2010b; Martins *et al.*, 2010). This technique consists in the penetration of microwave energy into the material structure, which produces a volumetrically distributed heat source due to molecular friction resulting from dipolar rotation of polar solvents and from the conductive migration of dissolved ions, accelerating the mass transfer of target compounds. In

general, the compounds are extracted more selectively and quicker by this technique, with similar or better yields in comparison with conventional extraction processes, using less energy and solvent volume, thus being more environmentally friend (Eskilsson and Björklund, 2000; Srogi, 2006; Bélanger and Paré, 2006). Only few works report the use of microwave-based techniques for extraction of compounds (alkaline galactans, carrageenans, and agar) from seaweeds (Uy *et al.*, 2005; Navarro *et al.*, 2007; Chhatbara *et al.*, 2009; Sousa *et al.*, 2010).

The present study evaluated the extraction of sulfated polysaccharides (fucoidan) from *Fucus vesiculosus* seaweed by MAE technique. An experimental design was applied to verify the influence of pressure, extraction time and alga/water ratio in the response of fucoidan yield, and the condition able to maximize the extraction yield was established. The percentage of alga degradation, total sugar yield in the hydrolysates after MAE, and SO_3 content were also determined to each experimental condition. Characterization of the recovered fucoidan was performed by HPLC, FTIR, and TGA/DSC analyses.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 CHEMICALS

Anthrone reagent was purchased from Prolabo, *Normapur*, Merck; 3,5-dinitrosalicylic acid from Fluka, Chemika, and Coomassie Plus (Bradford) assay kit was from Thermo Scientific Co. Other reagents were all of analytical grade.

4.2.2 ALGA COLLECTION AND SAMPLE PREPARATION

Fucus vesiculosus seaweed was collected from the Praia Norte, Viana do Castelo, Portugal, during September 2009. After collected the algal material was washed with fresh water in order to remove salt, sand and epiphytes, dried at 35 ºC, and milled using a home blender. Particles lower than 1000 µm were not used in experiments. Milled material was kept in plastic bags at room temperature for use in the extraction

experiments. Material samples were analyzed to determine the moisture and ash contents (AOAC official methods). The total sugars content present in the alga composition was determined after sulfuric acid hydrolysis for 2 h under vigorous agitation.

4.2.3 EXTRACTION PROCEDURE

MAE experiments were performed in a digestion oven model MDS-2000 (CEM Corporation, Matthews, NC). For each experiment, reaction vessels interconnected with tubing were placed in the sample holder, a rotating carousel. One of the vessels was equipped with pressure sensor that measured and controlled the set point within the cell.

For the extraction reactions, milled seaweed was suspended in the desired amount of distilled water and placed into the extraction vessel. The suspensions were irradiated under different pressures, for times varying between 1 and 31 minutes. Conditions of alga/water ratio, pressure and time used in each experiment are shown in Table 4.1. After irradiation, the vessels were immediately cooled in ice bath and the suspensions were filtrated through nylon fiber to separate the residual alga, which was dried at 35 °C, weighted to determine the residual amount obtained (value that was also used to calculate the alga degradation, % AD), and stored. An aliquot of each obtained hydrolysate was taken for total sugar quantification (% TS-A_{MAE}). Subsequently, 1% (w/v) $CaCl_2$ solution was added to the liquid fraction and the mixture was maintained overnight at 4 °C for alginate removal. The fraction obtained by ionization of $CaCl_2$ was separated by filtration. Double volume of ethanol absolute was added to the resultant filtrate and the mixture was stored at 4 °C for 8 h. Ethanol-precipitated polysaccharide was recovered by centrifugation (8,500 rpm, 15 min, 4 °C), dried at 35 ºC, milled and stored for further analyses.

Table 4.1. Experimental conditions used for MAE of *Fucus vesiculosus* according to a 2^3 full experimental design. Real and (coded) values of the operational variables pressure (x_1), extraction time (x_2) and alga/water ratio (x_3), and results obtained for the responses fucoidan yield (Y_1; % Fuc), alga degradation (Y_2; % AD), total sugar yield of hydrolysates after MAE (Y_3 ; % TS-A_{MAE}), and sulfate content (Y_4; % SO_3).

	Variables [a]						Responses			
Assay	x_1		x_2		x_3		Y_1 (% Fuc)	Y_2 (% AD)	Y_3 (% TS-A_{MAE}) [b]	Y_4 (% SO_3)
1	30	(-1)	1	(-1)	1/25	(-1)	6.25	28.82	9.42	20.08
2	30	(-1)	1	(-1)	5/25	(+1)	1.08	27.92	1.39	16.87
3	30	(-1)	31	(+1)	1/25	(-1)	15.61	48.99	24.52	22.76
4	30	(-1)	31	(+1)	5/25	(+1)	8.60	42.57	3.59	27.63
5	120	(+1)	1	(-1)	1/25	(-1)	18.22	51.36	27.62	21.09
6	120	(+1)	1	(-1)	5/25	(+1)	10.93	46.33	4.39	24.88
7	120	(+1)	31	(+1)	1/25	(-1)	6.93	67.98	25.54	30.31
8	120	(+1)	31	(+1)	5/25	(+1)	5.74	42.59	3.68	35.55
9	75	(0)	16	(0)	3/25	(0)	12.53	48.76	9.65	23.07
10	75	(0)	16	(0)	3/25	(0)	13.24	50.51	10.01	22.57
11	75	(0)	16	(0)	3/25	(0)	12.16	47.02	8.56	24.99
12	75	(0)	16	(0)	3/25	(0)	12.36	51.40	11.36	22.33

[a] pressure (x_1): psi; time (x_2): min; alga/water ratio (x_3): g mL^{-1}.

[b] % TS-A_{MAE} was calculated by the ratio between mg of total sugars in the hydrolysates obtained after MAE, and mg of total sugars in the alga (35.12 mg/100 mg).

Fucoidan extraction yield (% Fuc), alga degradation (% AD), and total sugar yield of hydrolysates after microwave-assisted extraction (% TS-A_{MAE}), were calculated according to Eq.1-3, where WM_{OH} is the dry mass weight obtained after ethanol precipitation; WA is the alga weight used in each experiment; WA_{MAE} is the dry alga weight recovered after MAE; TS_H_{MAE} is the mg of total sugars in the hydrolysates obtained after MAE; and TS_A is the mg of total sugars in the alga *Fucus vesiculosus* (35.12 mg TS/100 mg alga).

$$\%Fuc = \frac{WM_{OH}}{WA} \times 100 \quad (1)$$

$$\%AD = \left(\frac{WA - WA_{MAE}}{WA} \right) \times 100 \quad (2)$$

$$\%TS - A_{MAE} = \left(\frac{TS\_H_{MAE}}{TS\_A} \right) \times 100 \quad (3)$$

4.2.4 CHARACTERIZATION OF THE RECOVERED FUCOIDAN

A mass of 10-15 mg of the recovered fucoidan was submitted to hydrolysis with 4 N HCl (2 mL) at 121 °C for 2 h. After the hydrolysis reaction, the total sugar content in the liquid fraction was determined by the anthrone method (using glucose as standard), and the content of sulfate groups was determined by turbidity through the barium chloride–gelatin method (Dodgson, 1961). All absorbance measurements were performed in triplicate.

For the determination of monosaccharides content by HPLC, 10-15 mg of the recovered fucoidan was hydrolyzed with 2 M trifluoroacetic acid (0.5 mL) at 121 °C for 2 h, in glass tubes sealed with N_2. After reaction, the tubes were cooled in ice-water bath, centrifuged (5000 rpm, 5 min), and the liquid fraction was neutralized to pH 7 with 2 M NaOH. Resulting samples were then injected into the HPLC system. A Jasco chromatograph system equipped with a refraction-index detector and a MetaCarb 87P (300 × 7.8 mm) column at 80 °C was used for the sugars determination. Deionized water was used as mobile phase at a flow rate of 0.4 mL min^{-1}.

Micrographs of seaweed samples before and after extraction were obtained by scanning electron microscopy using a Nova NanoSEM 200 microscope. For the analyses, the samples were fixed on a specimen holder with aluminum tape and then sputtered with gold in a sputter-coater under high vacuum condition. Images were obtained at magnification of 2000 fold.

Thermal gravimetric analysis (TGA) data were taken with a thermo balance model TGA-50 (Shimadzu Corporation, Kyoto, Japan) in a nitrogen atmosphere. Differential scanning calorimetry (DSC) analyses were performed using a Modulate DSC-50 (Shimadzu Corporation, Kyoto, Japan). Mass samples of 10-13 mg were run from room temperature to 600 °C, at a rate of 10 °C min^{-1}.

Infrared analysis spectroscopy (FTIR) was carried out on a Perkin-Elmer 16 PC spectrometer (Boston, USA) using 16 scans and frequency range of 400-4000 cm^{-1}. For FT-IR measurement, the polysaccharide was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets. The vibration transition frequencies of each spectrum were baseline corrected and the absorbance was normalized between 0 and 1.

4.2.5 EXPERIMENTAL DESIGN

A 2^3 full experimental design with four replicates at the centre point was used to evaluate the effects of the variables pressure (X_1; psi), time (X_2; min), and alga/water ratio (X_3; g mL^{-1}) on the extraction of fucoidan under MAE conditions. For statistical analysis, the variables were coded according to Eq. 4, where each independent variable is represented by x_i (coded value), X_i (real value), X_0 (real value at the centre point), and ΔX_i (step change value). The real and coded values of the variables are given in Table 4.1. Low and high factors were coded as -1 and +1; the centre point was coded as 0.

$$x_i = (X_i - X_0) / \Delta X_i \quad (4)$$

Four assays at the centre point of the design were carried out to estimate the random error needed for the analysis of variance, as well as to examine the presence of

curvature in the response surfaces. The fucoidan yield (Y_1; % Fuc), alga degradation (Y_2; % AD), total sugar yield of hydrolysates after MAE (Y_3; % TS-A_{MAE}), and the sulfate content (Y_4; % SO_3) were taken as dependent variables or responses of the experimental design. The results were analyzed by analysis of variance (ANOVA), and the responses and variables (in coded unit) were correlated by response surface analysis to obtain the coefficients of Eq. 5.

$$Y_i = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 \quad (5)$$

In Eq. 5, Y_i represents the response or dependent variable; a_0 is the interception coefficient; x_1, x_2 and x_3 are the coded levels of the three variables (pressure, time and alga mass/water volume ratio), and a_1, a_2, a_3, a_{12}, a_{13}, a_{23} are the regression coefficients. The statistical significance of the regression coefficients was determined by Student's *t*-test, and the proportion of variance explained by the models was given by the multiple coefficient of determination, R^2. Statistica 5.0 was the software used for data analysis.

4.3 RESULTS & DISCUSSION

4.3.1 ALGA CHARACTERIZATION

Fucus vesiculosus contained a moisture content of 15.95 ± 0.08% (w/w). This value is higher than those reported to other marine algae such as *Laminaria* (6.64%) and *Gigartina* (9.86%) (Gómez-Ordóñez *et al.*, 2010), and is a positive aspect considering the alga use in MAE because the moisture content is closely related to the absorption efficiency of microwaves by the immersed target material. The water molecules convert the microwave energy into heat, resulting in a sudden rise of the temperature inside the material. The temperature keeps rising until the internal pressure exceeds the capacity of expansion of the matrix thus creating an explosion at the intermolecular level. As a consequence, the substances that are located within these chemical systems migrate to the surrounding medium that traps and dissolves them (Bélanger and Paré, 2006).

Ashes in *Fucus vesiculosus* corresponded to 18.32 ± 0.83% (w/w), a high value currently found in seaweeds, but much higher than those generally observed in terrestrial vegetables. Ashes content comprises the minerals present in the material. Although the minerals present in *Fucus vesiculosus* ashes were not determined here, brown seaweeds have been reported to have high chloride content, small amounts of fluoride, nitrate and phosphate, and trace amounts of nitrite and bromide. Due to the significant mineral content present in their chemical composition, several seaweeds have been used as food supplement to help meet the recommended daily intakes of some minerals and trace elements (Gómez-Ordóñez *et al.*, 2010)

Total sugars content in *Fucus vesiculosus* was 35.12 ±0.02% (w/w). This value is lower than those reported by Rioux *et al.* (2007) for brown seaweeds, and probably, it is a consequence of the period in which the alga was harvested. Algae generate their biomass reserve after the rapid grow phase in spring in order to survive the winter where hardly any photosynthesis occurs. As a consequence, a larger amount of polysaccharides is found during the winter season. In the present study, the alga was harvested in September (autumn season), which was not the best collection period.

4.3.2 OPERATIONAL VARIABLES AFFECTING FUCOIDAN EXTRACTION BY MAE

Several studies report MAE as a technique able to produce biopolymers with high molar mass at significantly shorter heating times than conventional extraction methods (Chen *et al.*, 2005; Leonelli and Mason, 2010). Considering this aspect and the structural and chemical complexity of sulfated polysaccharides, MAE was used in the present study to extract fucoidan from algal material. It was also expected that by using this method fucoidan would undergo degradation. In this work the microwave energy over the target material was controlled under pressure parameter, because one of the most frequent problems of heating by microwave fields is the temperature measurements, which are complicated by the presence of high intensity electromagnetic fields (Kustov and Sinev, 2010).

The used extraction conditions, including pressure, extraction time and algae/water ratio were selected based on previous studies for the production of other heteropolymers by MAE, such as pectin from citric peels or sugar beet pulp (Fishman *et al.*, 2000; Fishman *et al.*, 2008). Pressure conditions, particularly, were evaluated until the maximal operational value allowed by the equipment. Figure 4.1 shows the pressure profiles against heating time of microwave irradiation for a sample load of 1 g per 25 mL of water.

Heat stages rates were estimated measuring the ramp up and ramp down through the heating and cooling phases between the isothermal periods of the extraction procedures. The equivalent temperature used to each pressure (after the system has reached a saturated vapor behavior) was estimated using tables of water liquid-vapor phase and corresponded to 122, 152 and 172 °C for 30, 75, and 120 psi, respectively. As can be seen in Figure 4.1, the samples reached the hydrothermal stage (constant pressure) in less than 2 min, showing similar pressure increment with heating rates of around 103-128 °C min^{-1}. On the contrary, the pressure reduction showed that cooling rates were dependent of the quantity of time that the sample was irradiated at the isothermal stage. As a consequence, the compounds hydrolysis is also influenced at the cooling phase.

Moreover, the time required to attain the desired pressure was also dependent of the number of vessels processed simultaneously in the equipment, and therefore, the number of vessels should be chosen in order to minimize the time needed to reach the set conditions and to avoid a ‘‘bumping’’ phenomenon during the extraction (Eskilsson and Björklund, 2000). The solid/liquid ratio, i.e., the ratio between alga mass and water volume used for the reactions, is also an important parameter to be considered in MAE. The product recovery by conventional extraction methods is usually increased when using high solvent volumes (Eskilsson and Björklund, 2000); however, similar behavior may not occur in MAE. For this reason, different solid/liquid ratios varying from 1/25 to 5/25 g mL^{-1} were evaluated in the present study. Table 4.1 shows the conditions of pressure, reaction time and alga/water ratio used in each experimental MAE assay, and the respective fucoidan yield, alga degradation, total sugar yield and sulfate content obtained. Great variation in all the responses was observed according to the used

experimental condition. Fucoidan yield, for example, was increased in up to 17 times, by varying the MAE conditions.

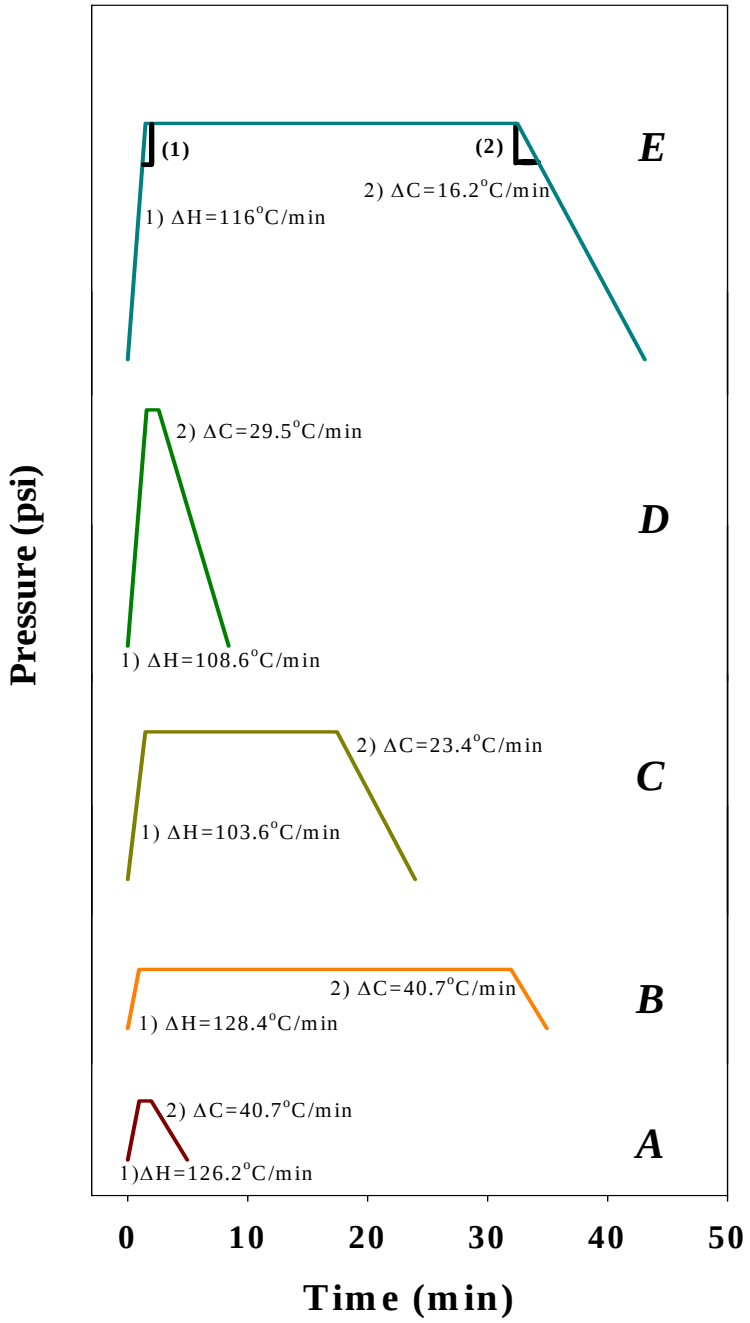

Figure 4.1. Pressure profiles as a function of radiation time during MAE of *Fucus vesiculosus* for fucoidan recovery; relation between heating and cooling rates (°C/min).
Sample load: 1 g alga/ 25 mL water. A) 30 psi (122 °C) / 1min; B) 30 psi (122 °C) / 31min; C) 75 psi (152 °C) / 16min; D) 120 psi (172 °C) / 1min; and E) 120 psi (172 °C) / 31min.
ΔH: heat rate (1); ΔC: cool rate (2).

All the studied operational variables affected the extraction process, presenting significant main effects and/or interactions for all of the evaluated responses (Table 4.2). For fucoidan yield, the alga/water ratio presented a significant main effect (p<0.05) of negative signal, which reveals that the fucoidan yield was increased when using an alga/water ratio of 1 g/25 mL.

Although the pressure and extraction time have not shown significant main effects for fucoidan yield, interaction between these variables was highly significant (p<0.01) for this response. When observing the main effects of these two variables for the other responses, it can be observed that pressure had a significant main effect of positive signal for all of them, which suggests that the extraction results were improved when the pressure was increased. As a consequence, since the interaction between pressure and reaction time had a significant negative effect for fucoidan yield response, it can be concluded that the use of lower reaction times favored the extraction process. This analysis is in agreement with the results presented in Table 4.1, which shows that the highest fucoidan yield (18.22%) was obtained when the highest pressure (120 psi) and the lowest extraction time (1 min) and alga/water ratio (1 g/25 mL) were used (conditions of the assay 5).

Similar behavior was reported by (Latha, 2007) during the biopolymers extraction by MAE. According to this author, the particle concentration increase promotes a strong absorption of the microwave energy near the surface of the vessel, and low penetration depth of microwave radiation, which reduces the percentage of extraction. On the other hand, the pressure increase promotes the temperature raise in a direct proportion. As a consequence, the extraction rate increases due to the viscosity and surface tension reduction (Eskilsson and Björklund, 2000).

In the present study, despite the pressure increase has favored the fucoidan yield, equipment limitations did not allow to evaluate pressure values higher than 120 psi. Additionally, the use of alga/water ratios lower than 1/25 g mL^{-1} might not be economically advantageous for the process since it would increase the costs for fucoidan recovery from the liquid phase.

Table 4.2. Effect estimates (EE), standard errors (SE) and level of significance (*p*) for fucoidan yield (Y_1; % Fuc), alga degradation (Y_2; % AD), total sugar yield of hydrolysates after MAE (Y_3; % TS-A_{MAE}), and sulfate content (Y_4; % SO_3) obtained after MAE of *Fucus vesiculosus* according to a 2^3 full experimental design.

Variables	Y_1 (% Fuc)		Y_2 (% AD)		Y_3 (% TS-A_{MAE}) [a]		Y_4 (% SO_3)	
	EE ± SE	*p*	EE ± SE	*p*	EE ± SE	*p*	EE ± SE	*p*
x_1	2.57 ± 1.99	0.2521	14.99 ± 3.19	0.0053 ***	5.58 ± 2.19	0.0512 *	6.12 ± 1.31	0.0055 ***
x_2	0.10 ± 1.99	0.9618	11.93 ± 3.19	0.0134 **	3.63 ± 2.19	0.1580	8.33 ± 1.31	0.0014 ***
x_3	-5.17 ± 1.99	0.0482 **	-9.44 ± 3.19	0.0315 **	-18.51 ± 2.19	0.0004 ***	2.67 ± 1.31	0.0970
x_1x_2	-8.34 ± 1.99	0.0085 ***	-5.49 ± 3.19	0.1460	-5.02 ± 2.19	0.0700 *	1.61 ± 1.31	0.2733
x_1x_3	0.93 ± 1.99	0.6609	-5.78 ± 3.19	0.1298	-4.03 ± 2.19	0.1245	1.84 ± 1.31	0.2188
x_2x_3	1.07 ± 1.99	0.6147	-6.47 ± 3.19	0.9816	-2.88 ± 2.19	0.2446	2.38 ± 1.31	0.1288

Significance level: 99% (***); 95% (**); 90% (*). x_1: pressure (psi); x_2: time (min); x_3: alga/water ratio (g mL^{-1}).

[a] % TS-A_{MAE} was calculated by the ratio between mg of total sugars in the hydrolysates obtained after MAE, and mg of total sugars in the alga (35.12 mg/100 mg).

Therefore, the optimal MAE conditions for fucoidan extraction from *Fucus vesiculosus* were established in the studied range of operational values. An analysis of variance of the obtained data for linear models gave high values for the coefficient of determination R^2 (between 0.84 and 0.95), which show a close agreement between experimental results and the theoretical values predicted by the first-order polynomials. A multiple regression analysis was then performed to fit first-order polynomial equations to the experimental data points. The fucoidan yield (Y_1, %), alga degradation(Y_2, %), total sugar yield of hydrolysate (Y_3, %), and the sulfate content (Y_4, %) were correlated as a function of extraction pressure (x_1), time (x_2) and alga/water ratio (x_3) (coded values) used for MAE, resulting in Eqs. 6, 7, 8, and 9, respectively.

$$Y_1 = 10.30 + 1.29x_1 + 0.05x_2 - 2.58x_3 - 4.17x_1x_2 + 0.46x_1x_3 + 0.53x_2x_3 \quad (6)$$
$$(R^2 = 0.84)$$

$$Y_2 = 46.19 + 7.50x_1 + 5.96x_2 - 4.72x_3 - 2.74x_1x_2 - 2.89x_1x_3 - 3.24x_2x_3 \quad (7)$$
$$(R^2 = 0.92)$$

$$Y_3 = 11.64 + 2.79x_1 + 1.81x_2 - 9.26x_3 - 2.51x_1x_2 - 2.02x_1x_3 - 1.44x_2x_3 \quad (8)$$
$$(R^2 = 0.95)$$

$$Y_4 = 24.34 + 3.06x_1 + 4.17x_2 + 1.37x_3 + 0.81x_1x_2 + 0.92x_1x_3 + 1.19x_2x_3 \quad (9)$$
$$(R^2 = 0.94)$$

Three-dimensional response surfaces described by the above-mentioned first-order polynomials were well fitted to the experimental data points through flat surfaces, confirming the suitability of the proposed linear models to explain the responses variations in the studied range of values. Figure 4.2 represents the variations in fucoidan yield according to the pressure and alga/water ratio used for extraction. As can be seen, the flat surface clearly indicates a region where the value of this response is maximized, which corresponds to the use of 120 psi, and 1/25 alga/water ratio (g mL^{-1}) during 1 min of extraction. The highest fucoidan extraction yield (18.22% in a dry weight basis) is in good agreement with the values reported by Rioux *et al.* (2007) during the extraction of *F. vesiculosus* by 3 sequential hydrolysis steps (each one of 3 h) at 70 °C. Moreover, this value was higher than those reported for fucoidan obtained from other sources

extracted by hydrothermal conventional procedures under temperatures between 25 and 70 °C and times of 2-6 h (Zvyagintseva *et al.* 1999; Duarte *et al.*, 2001; Navarro *et al.*, 2007). Additionally, Yang *et al.* (2008) evaluated the hydrolysis of sulfated polysaccharides of *U. pinnatifida* testing twice microwave for 30-120 sec and founded that microwave heating around 30-60 seconds only was more effective in improving the polymer dissolution.

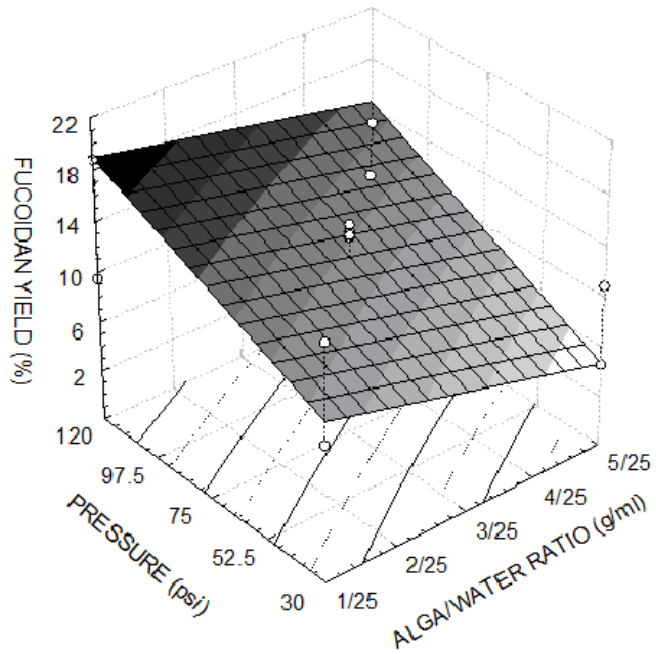

Figure 4.2. Response surface fitted to the experimental data points corresponding to the fucoidan yield during MAE of *Fucus vesiculosus*.

Figure 4.3 shows the alga structure before and after MAE under optimum conditions. As can be seen, the untreated sample (Figure 4.3A) presented closed cells and rough surfaces, which were mostly destroyed after MAE (Figure 4.3B). A less destructive effect of destruction in the alga structure was observed after MAE under milder pressure conditions (Figure 4.3C). Such facts evidence the importance of the pressure increase on the extraction process, as commented before. The alga structure after MAE under high pressure (120 psi, Figure 4.3B) was formed by a very rough surface with many cavities, suggesting that microwave radiation had the power on cuticular layer destruction, as observed also by other authors (Chen *et al.*, 2005).

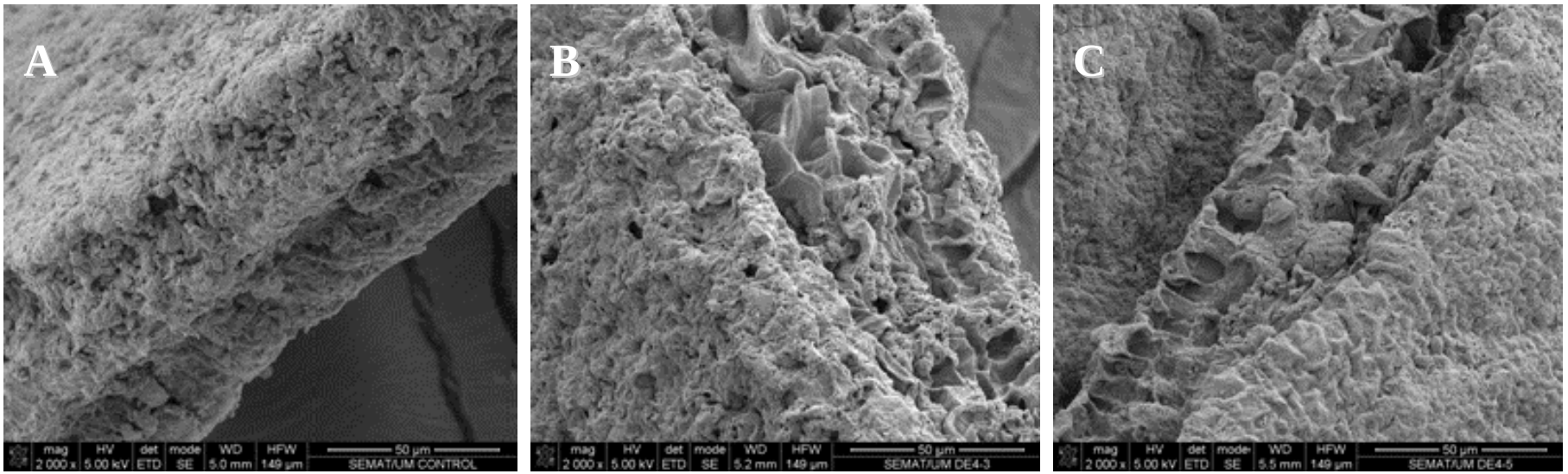

Figure 4.3. Scanning electron micrographs of *Fucus vesiculosus*: (A) untreated sample; (B) sample obtained after MAE at 120 psi, 1 min, using 1 g alga/ 25 mL water; (C) sample obtained after MAE at 30 psi, 31 min, using 1 g alga/ 25 mL water. Magnification: 2000-fold.

4.3.3 CHARACTERIZATION OF THE EXTRACTED FUCOIDANS

4.3.3.1 Compositional analysis

The fucoidans obtained in all the experimental MAE conditions were characterized regarding the monosaccharide and sulfate contents (Table 4.3). L-Fucose was the only monosaccharide found in all the samples. Galactose was also present in most of the samples, but xylose was only present in some of them. The results presented in Table 4.3 suggest that the pressure used for extraction had a strong influence on the fucoidan composition, since the galactose contents in the fucoidan structure were increased when the pressure used for extraction was increased to 120 psi; and only fucose was present in the fucoidans obtained at 30 psi. Similarly, xylose was only present in structures obtained at 120 psi. Under the optimum MAE conditions, a fucoidan structure composed predominantly by fucose, followed by significant proportion of xylose and minor galactose content was obtained (Table 4.3, assay 5). This is in agreement with literature data that report that fucoidan from *F. vesiculosus* has a heterogeneous and branched structure (Marais and Joseleau, 2001).

Besides the monosaccharide content, the conditions used for MAE affected also the fucoidans sulfating degree (Table 4.3). However, high sulfate content (> 20%) was found in practically all the fucoidan samples, which is an advantageous aspect since sulfate groups have been reported to have important biological functions such as anti-HIV activity; and such activity is potentially increased when the sulfating degree is increased (Schaeffer and Krylov, 2000). Additionally, the presence of non-sulfate monosaccharide units in polysaccharides branches is reported to annul the anticoagulant effect of the polysaccharide (Costa *et al.*, 2010). The ratio between total sugars and sulfate content (TS/SO_3, Table 4.3) is considered an indicator of the anticoagulant activity of fucoidan polysaccharides (Wang *et al.*, 2008). In the present study, most of the experiments showed TS concentrations similar or higher than SO_3 concentrations.

Table 4.3. Monosaccharide and sulfate composition of fucoidan isolated from *Fucus vesiculosus* by MAE under different operational conditions according to a 2^3 full experimental design. Monosaccharide amount are expressed as the percent of the total sugar content in the sample, in moles.

Assay	Pressure (psi)	Extraction time (min)	Alga/water ratio (g mL^{-1})	Fucose (% mol)	Galactose (% mol)	Xylose (% mol)	TS/SO_3*
1	30	1	1/25	100.0	0.0	0.0	1/1.00
2	30	1	5/25	100.0	0.0	0.0	1/0.89
3	30	31	1/25	100.0	0.0	0.0	1/0.89
4	30	31	5/25	82.3	17.6	0.0	1/1.07
5	120	1	1/25	53.8	10.8	35.3	1/0.77
6	120	1	5/25	57.4	42.5	0.0	1/0.96
7	120	31	1/25	27.1	42.9	29.9	1/1.84
8	120	31	5/25	39.1	60.8	0.0	1/2.11
9	75	16	3/25	49.0	50.9	0.0	1/1.12
10	75	16	3/25	49.8	50.1	0.0	1/0.93
11	75	16	3/25	53.6	46.3	0.0	1/0.96
12	75	16	3/25	57.6	42.3	0.0	1/1.02

* TS/SO_3 = (mg TS/100 mg fucoidan)/(mg SO_3/100 mg fucoidan). TS: total sugars.

Fucoidan polymers from other sources had comparable amounts of sulfates (19–30%) and monosaccharide composition with fucose as the major sugar in the extracted fucoidans (50-90 % mol) and lower amounts of galactose and xylose (Zvyagintseva *et al.*, 1999; Duarte *et al.*, 2001; Rioux *et al.*, 2007). However, it is important emphasizing that chemical composition of fucoidan polymers is significantly dependent on species, anatomical regions, growing conditions, extraction procedures and analytical methods.

4.3.3.2 Thermal analysis

TGA and DSC curves of fucoidan extracted under optimum MAE conditions are showed in Figure 4.4.

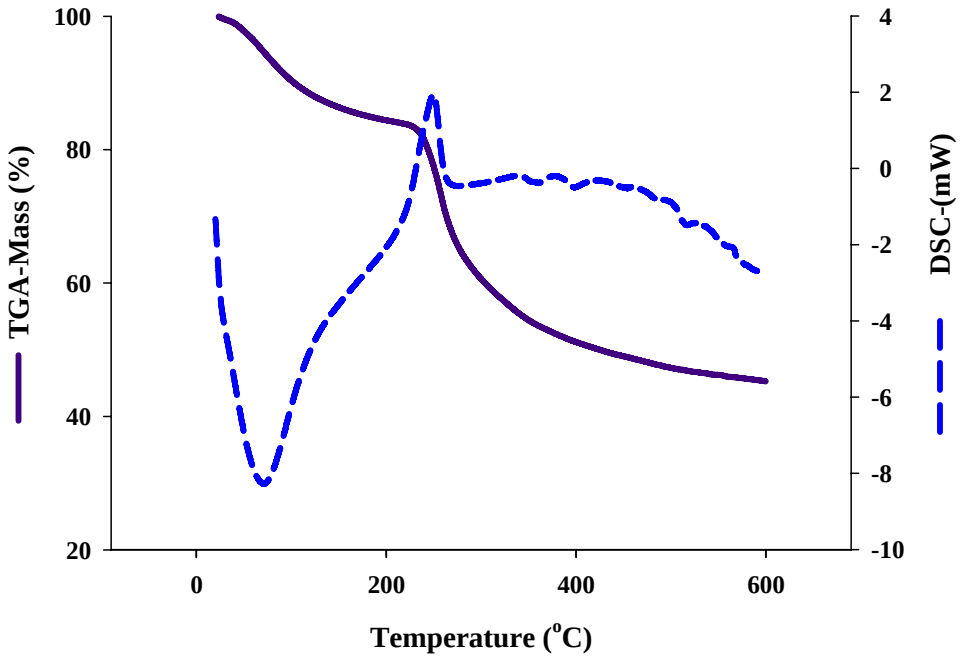

Figure 4.4. TGA and DSC thermograms of fucoidan sample obtained under optimum MAE conditions.

Three different stages were well defined during these analyses. The first one was basically associated with the weight loss (moisture) due to dehydration, which covered a temperature range between 25 °C and 110 °C. Subsequently, pyrolysis reactions of the

sample started at 120 °C. The second stage started at 195 °C and consisted in the devolatilization of the sample, with evolution of the volatile matter mainly occurring between 220 °C and 490 °C. Finally, the third stage began close to 500 ºC and was maintained up to 600 °C. The remaining mass at the end of this process (around 50% of the original fucoidan mass) corresponds to the ash content in the sample. This residual mass is probably constituted by sulfates, phosphates and carbonates, which are minerals usually found in polysaccharides structures like fucoidan (Anastasakis *et al.*, 2011).

4.3.3.3 FTIR analysis

Fucoidan obtained under optimum MAE conditions, as well as fucoidan samples obtained under other evaluated extraction conditions, were analyzed by FTIR to determine the specific absorption bands present in the recovered products. The FTIR spectra in Figure 4.5 clearly show that all the evaluated samples exhibited absorption bands typical of fucoidans.

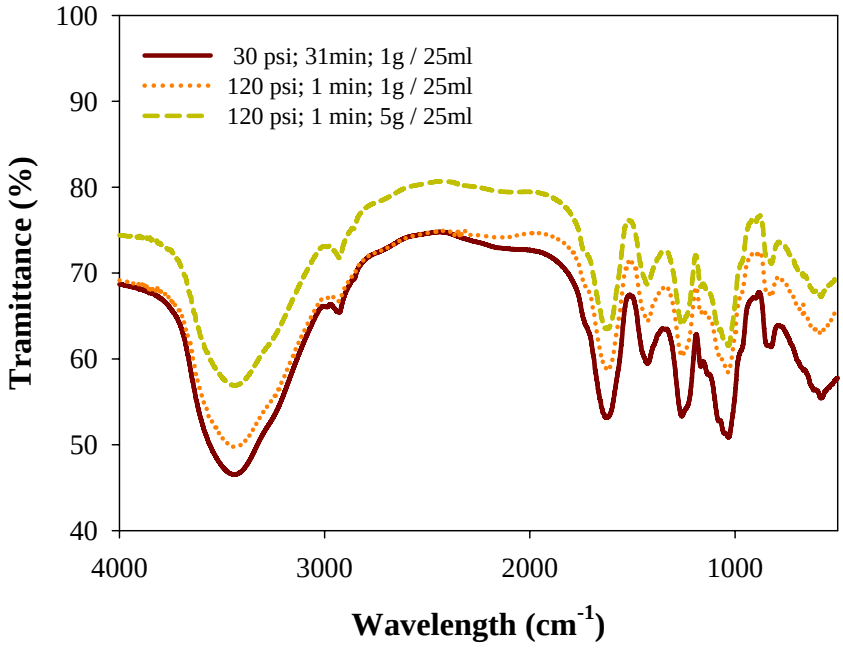

Figure 4.5. Infrared analysis spectroscopy (FTIR) of fucoidan samples obtained by MAE of *Fucus vesiculosus* under different operational conditions.

The absorption band at 1240–1255 cm^{-1} (S=O stretching) confirmed the presence of sulfate in the recovered polysaccharides. The sharp band at 840 cm^{-1} and the shoulder at 820 cm^{-1} (C-S-O) suggest a complex pattern of substitution, primarily at C-4 position (axial C-4 substitution of α-linked L-fucopyranose) with other substitution at C-2 or/and C-3 (equatorial positions) in lower amount (Marais and Joseleau, 2001; Wang *et al.*, 2010a).

4.4 CONCLUSIONS

In summary, MAE under optimum reaction conditions was an effective method to recover fucoidan from *Fucus vesiculosus*. This method required short extraction time and use of non corrosive solvents, resulting in reduced costs when compared to the conventional extraction techniques. Additionally, MAE can be considered a more environmentally friend technique than the traditional extraction processes, since it requires lower energy consumption and generates less wastes. For all these reasons, MAE was considered a potential method to obtain fucoidan from brown seaweed.

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CHAPTER 5

EXTRACTION OF SULFATED POLYSACCHARIDES BY AUTOHYDROLYSIS OF BROWN SEAWEED *FUCUS VESICULOSUS*

ABSTRACT

The extraction of sulfated polysaccharides (fucoidan) by autohydrolysis (AH) of brown seaweed *Fucus vesiculosus* was studied. Experimental assays were performed under different conditions of temperature (160 to 200 °C) and reaction time (10 to 30 min) according to a 2^2 central composite design (CCD), and those able to maximize the fucoidan yield were selected. The alga degradation and the total sugar yield in the liquor after AH were also determined to each experimental condition. The highest fucoidan yield (~16.5% w/w) was obtained when the AH process was performed at 180 °C for 20 min. This product was characterized by HPLC, FTIR, and thermal gravimetric analyses, being verified the presence of fucose and galactose as main components (70:30% mol ratio), and an SO_3 content higher than 20%. AH process under optimum reaction conditions was an effective and environmentally friendly method to recover fucoidan from *Fucus vesiculosus*.

5.1 INTRODUCTION

In the recent years, great attention has been given to the use of marine seaweeds biomass. Such interest has been supported by important advantages that the use of this kind of biomass represents, including: a) low future fluctuations in biomass demand are expected due to overpopulation; b) feasibility of growing fast in the open ocean; c) higher photosynthetic activity (6–8%) than terrestrial biomass (1.8–2.2%); d) no limitation by water and to a lesser extent by temperature; and e) low costs of collection and null environmental damage (Anastasakis *et al.*, 2011; Ross *et al.*, 2008). Main components of marine seaweeds differ from that of terrestrial biomass (cellulose, hemicellulose, and lignin), and include phytochemically active molecules such as polysaccharides, fatty acids, proteins, vitamins, and mineral elements, which are compounds with potential applications in food, cosmetic, pharmaceutical, and medical fields (Anastasakis *et al.*, 2011; Lordan *et al.*, 2011; O'Sullivan *et al.*, 2010).

Brown seaweed derived polysaccharides such as fucoidan, laminaran, alginates, and mannitol have been studied due to their effectiveness as anticoagulant, antitumor, antithrombotic, anti-inflammatory, contraceptive, and antiviral agent (Bhakuni and Rawat, 2005; Imbs *et al.*, 2009; Mestechkina and Shcherbukhin, 2010; Wang *et al.*, 2009). Fucoidans, or sulfated fucans, may constitute up to 25–30% of the seaweed dry weight, depending on the seaweed species and, to a lesser extent, on season (Skriptsova *et al.*, 2010). The most relevant biological functionalities of these compounds are the activities against hepatitis, herpes and human immunodeficiency (AIDS) viruses, anticoagulant heparin inflammation, cell proliferation and adhesion, and fertilization functions (Berteau and Mulloy, 2003; Ellouali *et al.*, 1993; Queiroz *et al.*, 2008).

The extraction of sulfated polysaccharides from seaweeds have been usually performed by using hot water, dilute acid, or dilute alkali, all of these methodologies involving long extraction time and high volume of diluents (Duarte *et al.*, 2001; Marais and Joseleau, 2001; Rioux *et al.*, 2007; Skriptsova *et al.*, 2010; Wang *et al.*, 2009; Yang *et al.*, 2008). Among the existing technologies of hydrolysis, the autohydrolysis (AH) is an eco-friendly process that could be an interesting alternative for application on the recovery of biological compounds from seaweeds. This process requires only the use of

water as extraction solvent, and the hydronium-catalyzed reactions of the material fibers proceeds through water autoionization at elevated temperatures (150–230 °C). This process offers several important advantages, such as: 1) simple and economical operation; 2) elimination of corrosive problems; 3) mild operational conditions for selective degradation of the biomass; 4) generation of low concentrations of sugar degradation products in the media (Garrote *et al.*, 1999; Ruiz *et al.*, 2011).

Although all the above mentioned benefits, research on the application of AH process to macroalgae is limited. The aim of the current study was to evaluate the extraction of sulfated polysaccharides (fucoidan) by AH of brown seaweed *Fucus vesiculosus*. An experimental design was proposed to evaluate the effect of the process variables (temperature and reaction time) on the responses: fucoidan yield, alga degradation, and total sugar yield in the liquor after extraction. The product obtained was characterized to determine the monosaccharides and sulfate contents. Infrared analysis spectroscopy and thermal gravimetric analyses were also performed to explain the characteristics of the extracted fucoidan.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 CHEMICALS

3,5-Dinitrosalicylic acid was purchased from Fluka, Chemika; anthrone reagent was from Prolabo, *Normapur*, Merck; and Coomassie Plus (Bradford) Assay Kit was obtained from Thermo Scientific Co. Other reagents were all of analytical grade.

5.2.2 ALGAL MATERIAL

Samples of *Fucus vesiculosus* were collected from Praia Norte (Viana do Castelo, Portugal), during the spring season (May, 2010). The seaweed was washed with fresh water in order to remove salt, sand, and epiphytes, dried at 35 ºC, milled in a home blender, and stored in plastic bags at room temperature. Particles lower than 1000 µm

were not used in the experiments. The seaweed sample had moisture and ash contents of 14.84 ± 0.83% (w/w) and 17.5 ± 0.25% (w/w), respectively (dry weight basis).

5.2.3 AUTOHYDROLYSIS PROCESS

Hydrothermal processing was performed in 160 ml total volume stainless steel cylinder reactors and then submerged in an oil bath with open heating circulator (Julabo Labortechnik GmbH, Seelbach, Germany) with PID temperature control (Ruiz *et al.*, 2012). For the extraction of sulfated polysaccharides from *Fucus vesiculosus*, 2 g of milled seaweed was suspended in 50 mL of distilled water (alga/water ratio of 1:25), and the mixture was placed in the cylinder reactors, which were submerged in an oil bath following the different conditions of temperature and reaction time showed in Table 5.1.

At the end of the reaction, the reactors were removed from the oil bath and immediately immersed in an ice bath to stop the reaction. The obtained suspension was vacuum-filtrated to separate the liquor from the residual alga, which was dried at 35 °C, weighted to determine the percentage of alga degradation ($\%AD_{AH}$), and stored. Total sugar yield in the liquor after AH ($\%TS_{LAH}$) was quantified, and subsequently, a 1% (w/v) solution of $CaCl_2$ was added to the liquor in a ratio of 1:1 (v/v) for alginate removal (4 °C overnight storage). Free alginate liquor was recovered by filtration in qualitative paper, and then double volume of ethanol absolute was added to the resultant filtrate, and the mixture was stored at 4 °C for 8 h. The precipitated polysaccharide ($\%Fuc_{AH}$) was recovered by centrifugation (8,500 rpm, 15 min, 4 °C), dried at 35 °C, milled, and stored for analyzes. Two replicates of each experiment were carried out.

The fucoidan yield ($\%Fuc_{AH}$), alga degradation ($\%AD_{AH}$), and total sugar yield in the liquor after extraction ($\%TS_{LAH}$) were calculated by using the Eq. (1)-(3), where WM_{OH} is the dry mass weight obtained after ethanol precipitation; WA is the alga weight used in each experiment; WA_{AH} is the dry alga weight recovered after AH; TS_{AH} is the mg of total sugar in the hydrolysates obtained after AH; and TS_A in the mg of total sugars in the alga *F. vesiculosus* (35.12 mg TS/ 100 mg alga).

$$\%Fuc_{AH} = \frac{WM_{OH}}{WA} \times 100 \quad (1)$$

$$\%AD_{AH} = \left(\frac{WA - WA_{AH}}{WA} \right) \times 100 \quad (2)$$

$$\%TS_{LAH} = \left(\frac{TS_{AH}}{TS_{A}} \right) \times 100 \quad (3)$$

The severity parameter ($\log R_0$) of the AH process was calculated by the Eq. (4) (Overend and Chornet, 1987), where t is the time (min) at the temperature of reaction T (°C), and 14.75 is an empirical parameter related with the temperature and activation energy.

$$R_0 = \int_0^t \exp\left[\frac{T(t) - 100}{14.75} \right] dt \quad (4)$$

In a subsequent step, sequential extraction assays were performed aiming to verify the possibility of reusing the residual alga (after the AH process) to maximize the fucoidan recovery yield. Sequential extraction procedures were performed under the previously established AH conditions (180 °C, 20 min), following the same procedure described above. After the first extraction (E1), the residual solid material was separated by filtration, resuspended in distilled water to reach the alga/water ratio of 1:25 (w/v), and hydrolyzed again (E2) in the oil bath system.

Table 5.1. Values of the process variables, severity factor (logR_0), and results of fucoidan yield (%Fuc_{AH}), alga degradation (%AD_{AH}), and total sugar yield in the liquor after extraction (%TS_{LAH}), to each experimental condition used for autohydrolysis of *Fucus vesiculosus*.

	Process variables – real and (coded) values						Responses		
Run	Temperature (ºC)		Reaction time (min)		logR_0 [a]	Final pH	%Fuc_{AH}	%AD_{AH}	%TS_{LAH}
1	200	(+1)	30	(+1)	4.4	4.90	4.58 ± 1.31	30.52 ± 1.45	14.49 ± 0.72
2	200	(+1)	10	(-1)	4.0	6.57	8.22 ±1.07	61.62 ± 5.39	8.62 ± 1.20
3	160	(-1)	10	(-1)	2.8	6.79	3.23 ± 0.08	73.82 ± 2.60	3.66 ± 0.17
4	160	(-1)	30	(+1)	3.2	6.31	6.05 ±1.14	61.42 ± 0.46	11.06 ± 2.16
5	180	(0)	10	(-1)	3.4	6.73	5.68 ± 0.32	66.32 ± 4.46	6.47 ± 0.72
6	180	(0)	30	(+1)	3.8	5.13	8.42 ± 1.49	34.09 ± 0.83	14.92 ± 0.12
7	200	(+1)	20	(0)	4.3	5.01	7.16 ± 0.87	32.67 ± 1.84	11.38 ± 1.67
8	160	(-1)	20	(0)	3.1	6.52	7.16 ± 0.86	61.55 ± 3.54	7.16 ± 0.31
9	180	(0)	20	(0)	3.7	6.15	18.14	47.35	13.35
10	180	(0)	20	(0)	3.7	6.20	16.57	45.77	14.89
11	180	(0)	20	(0)	3.7	6.09	15.34	43.67	13.98
12	180	(0)	20	(0)	3.7	6.41	15.88	44.25	15.89

[a] The severity parameter of the autohydrolysis process was calculated by Eq. (4).

5.2.4 ANALYTICAL PROCEDURES

For characterization of the recovered fucoidan, samples of 10-15 mg of the fucoidan extracts were hydrolyzed with 4 N HCl (2 mL) at 121°C for 2 h. The total sugar content was then measured by the anthrone method using glucose as standard (Ludwig and Goldberg, 1954), and the sulfate group content was determined by turbidity with barium chloride–gelatin method (Dodgson, 1961). All absorbance measurements were carried out in triplicate. Protein was determined by the method of Bradford; and the total phenolic compounds by the method of Folin-Ciocalteau.

The concentration of monosaccharides was determined by hydrolysis of the fucoidan samples (10-15 mg) with 2 M trifluoroacetic acid (0.5 mL) at 121 ºC for 2 h, in glass tubes sealed with N_2. Hydrolyzed polysaccharides were cooled in an ice-water bath, centrifuged at 5000 rpm for 5 min, and the liquid fraction was neutralized to pH 7 with 2 M NaOH. The resulting samples were injected in a high performance liquid chromatography (HPLC - Jasco, Tokyo, Japan) system equipped with a low-pressure gradient solvent pump, an autosampler with 20-µl loop, and a refraction-index detector (Jasco, Tokyo, Japan). Samples were injected in a MetaCarb 67H (300 × 7.8 mm) column at 60 °C, using 0.005 M H_2SO_4 as mobile phase at a flow rate of 0.5 mL/min.

Micrographs of the seaweed samples were obtained in a scanning electron microscope Nova NanoSEM 200 (Netherlands) using the samples sputtered with gold under high vacuum conditions, and an accelerating voltage of approximately 15 kV. Images were obtained at magnification of 5000 fold.

Thermal gravimetric analysis (TGA) was performed in a thermo balance model TGA-50 (Shimadzu Corporation, Kyoto, Japan) under nitrogen atmosphere. Differential scanning calorimetry (DSC) analysis was performed using a Modulate DSC-50 (Shimadzu Corporation, Kyoto, Japan). Mass samples of 10-13 mg were run from room temperature to 600 °C at a rate of 10 °C/min.

Infrared analysis spectroscopy (FTIR) was carried out on a Perkin-Elmer 16 PC spectrometer (Boston, USA), using 16 scans and frequency range of 400-4000 cm^{-1}. For the analysis, the polysaccharide was ground with potassium bromide (KBr) powder and then pressed into 1 mm pellets. The vibration transition frequencies of each spectrum were baseline corrected and the absorbance was normalized between 0 and 1.

5.2.5 EXPERIMENTAL DESIGN AND DATA ANALYSIS

The effects of the independent variables: temperature (X_1; °C) and residence time (X_2; min) on the extraction of fucoidan by AH were evaluated through a 2^2 central composite design (CCD) with four replicates at the centre point. The variables were coded according to Eq. (5), where x_i is the coded value of the variable X_i; X_0 is the value of X_i at the centre point; and ΔX_i is the step change. The real and coded values of the variables are shown in Table 5.1. Experimental runs were randomized to minimize the effects of unexpected variability in the responses.

$$x_i = (X_i - X_0) / \Delta X_i \quad (5)$$

The fucoidan yield (% Fuc_{AH}), alga degradation (%AD_{AH}), and total sugar yield in the liquor after extraction (%TS_{LAH}) were taken as responses of the experimental design. The results were analyzed by analysis of variance (ANOVA), and the behavior of the system was explained by the Eq. (6), where Y is the dependent variable, β_0 is constant, β_i, β_{ii}, and β_{ij} are the coefficients estimated by the model, and X_i and X_j are the levels of the independent variables. Analyses of the experimental data were carried out using the software STATISTICA™ v 6.0 (Statsoft®, Tulsa, OK, USA).

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \quad (6)$$

5.3 RESULTS & DISCUSSION

5.3.1 SULFATED POLYSACCHARIDES YIELDS

AH technique has been reported as a technology that has been conceived as an optimal first step for substrate pretreatment modification. The effects of the temperature and residence time were investigated in order to maximize the fucoidan yield, thus, the used extraction conditions were selected based in the previous study reported by Ruiz *et al.*, (2011).

The fucoidan yield ($\%Fuc_{AH}$), alga degradation ($\%AD_{AH}$), and total sugar yield in the liquor after extraction ($\%TS_{LAH}$) to each experimental AH condition are shown in Table 5.1. As can be seen, great variations occurred in all these responses according to the used process condition; and in order to compare the effects caused by AH conditions over sulphated fucans solubilization the value of the severity factor (R_0) in the different assays was calculated and the tendency of the studied variables was plotted in Figure 5.1. The fucoidan yield, for example, was increased when the value of the severity factor ($\log R_0$) was increased, but up to a certain limit [$\log R_0$= 3.7 (180 °C and 20 min; Runs 9-12) - $\%Fuc_{AH}$ = 15-18% w/w] after which a significant lost of yield was observed [$\log R_0$= 4.4 (200 °C and 30 min; Run 1) - $\%Fuc_{AH}$ = 4.6% w/w]. The pH decrease observed in the reaction media obtained by using the highest values of severity factor may have affected the fucoidan recovery, since acid media could have favored the hydrolysis of this polysaccharide, causing a decrease in the final recovery yield.

The highest values of total sugar yield in the liquor (> 14% w/w) were obtained when using values of $\log R_0$ similar or higher than 3.7. This result suggests that the sugars obtained at 180 °C for 20 min (runs 9-12) probably are polysaccharides of long chain able to agglomerate and precipitate as sulfated fucans, causing an elevated fucoidan recovery yield. On the contrary, it is also possible that the total sugars obtained at 200 °C for 30 min (run 1) are mainly monosaccharide and/or short chain polysaccharides, explaining the low recovery yield.

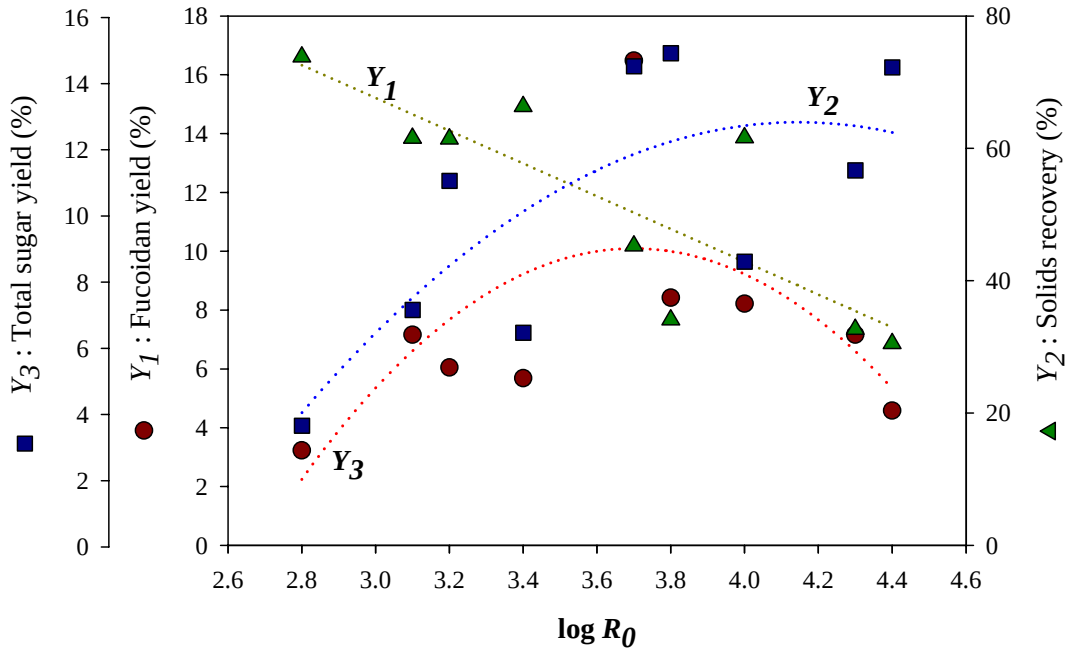

Figure 5.1. Severity parameter effect and tendency of studied variables after AH process: fucoidan yield (Y_1), solids recovery (Y_2) and total sugar yield (Y_3).

The effect of the AH process over brown seaweed fucoidan may be attributed to the heterogeneous backbone structure of this molecule, with alternating (1→3); (1→4)-linked 2- and/or 4-sulfated- α-L-Fucρ residues. The depolymerization of this structure affects the (1→3)-α-L-Fucρ residues faster than the (1→4)-α-L-Fucρ residues, causing intermolecular changes with transference of the sulfate groups to pentose and hexose sugars, which allow obtaining polysaccharides and oligosaccharides of multisulfated (up to 3) fucans (Anastyuk *et al.*, 2010). For this reason, all the fucoidan samples recovered in the current study presented high sulfate content (>18%).

The highest fucoidan yield (~16.5% w/w) obtained in the present study was similar to the values reported by Rioux *et al.* (2007), and Rodriguez-Jasso *et al.* (2011), by sequential hydrolysis and microwave assisted extraction of brown seaweeds, respectively; and was higher than the values reported for fucoidan obtained by AH of *Laminaria cichorioides* (Anastyuk *et al.*, 2010).

5.3.2 STATISTICAL ANALYSIS AND OPTIMIZATION OF AUTOHYDROLYSIS CONDITIONS

The effect estimates of the operational variables as well as their significance level on the responses are shown in Table 5.2. As can be noted, the studied variables significantly affected all the responses, presenting individual (first and second order) and/or interaction effects. A three dimensional response surface was plotted in order to represent the fucoidan yield variations as a function of the temperature and reaction time variations. This figure (Figure 5.2) clearly shows that the fucoidan yield was not linearly increased when the process variables were increased, but there was an optimum point after which the use of higher temperature and reaction time did not improve the yield. This is in agreement with the analysis presented in Table 5.2, which revealed significant effect of the quadratic term of both variables on the fucoidan yield response. An estimative of the critical point revealed that 180 °C and 20 min were the conditions able to maximize the fucoidan yield.

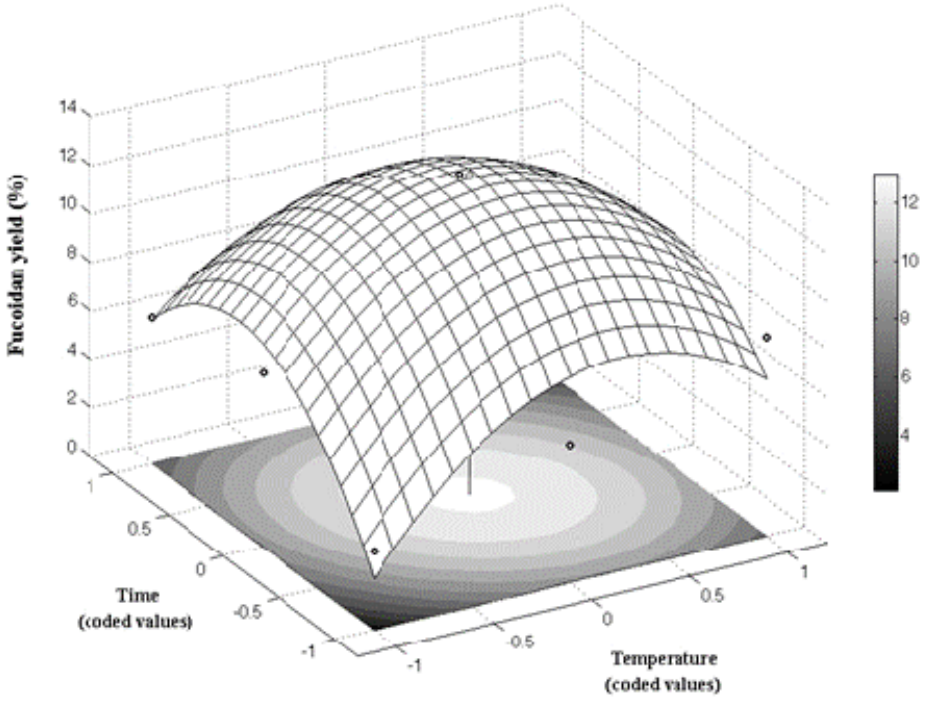

Figure 5.2. Response surface fitted to the experimental data points corresponding to the fucoidan yield during autohydrolysis of *Fucus vesiculosus* under different conditions of temperature and reaction time.

Table 5.2. Effect estimates (EE), standard errors (SE), and level of significance (*p*) for the responses of fucoidan yield ($\%Fuc_{AH}$), alga degradation ($\%AD_{AH}$), and total sugar yield in the liquor after extraction ($\%TS_{LAH}$), obtained by autohydrolysis of *Fucus vesiculosus*.

Variables	$\%Fuc_{AH}$		$\%AD_{AH}$		$\%TS_{LAH}$	
	EE ± SE	*p*	EE ± SE	*p*	EE ± SE	*p*
x_1	1.17 ± 2.34	0.634	-23.99 ± 2.94	0.000 ***	4.20 ± 1.31	0.019 **
x_1^2	-10.85 ± 3.51	0.021 **	8.49 ± 4.42	0.103	-6.50 ± 1.97	0.017 **
x_2	0.64 ± 2.34	0.793	-25.24 ± 2.94	0.000 ***	7.24 ± 1.31	0.001 ***
x_2^2	-11.07 ± 3.51	0.020 **	14.68 ± 4.42	0.016 **	-3.65 ± 1.97	0.114
$x_1 x_2$	-3.23 ± 2.87	0.303	-9.35 ± 3.60	0.041 **	-0.77 ± 1.61	0.651

Significance level: (**) 95%, (***) 99%; x_1: temperature, x_2: reaction time

The alga degradation and the total sugar yield in the liquor after extraction were also affected by the quadratic terms of the variables (Table 5.2). This fact suggests that, similarly to the observed for the fucoidan yield, the variation of these responses did not occur linearly by increasing the value of the variables. A regression analysis was then performed to fit equations able to predict the value of the responses according to the temperature and reaction time variations used in the present study. The mathematical models expressed in Eq. (7)–(9) (coded values of the variables) represent the fucoidan yield (Y_1), alga degradation (Y_2), and total sugar yield in the liquor after extraction (Y_3), as a function of the temperature (x_1) and reaction time (x_2) used during the AH. Such models were established with high coefficient of determination R^2 being able to explain between 84% and 97% of the variations in these responses.

$$Y_1 = 15.18 + 0.59x_1 - 5.43x_1^2 + 0.32x_2 - 5.54x_2^2 - 1.62x_1x_2 \qquad (R^2 = 0.84) \qquad (7)$$

$$Y_2 = 44.46 - 12.00x_1 + 4.25x_1^2 - 12.62x_2 + 7.34x_2^2 - 4.68x_1x_2 \qquad (R^2 = 0.97) \qquad (8)$$

$$Y_3 = 13.86 + 2.10x_1 - 3.25x_1^2 + 3.62x_2^2 - 1.82x_2^2 - 0.38x_1x_2 \qquad (R^2 = 0.91) \qquad (9)$$

Figure 5.3 shows the alga structure before and after the AH process under the optimized conditions (180 °C for 20 min). As can be seen, the original (untreated) sample exhibited a rigid and ordered surface (Figure 5.3A), which was modified after the AH process (Figure 5.3B), becoming more porous and rough due to the removal of components from this structure. The TGA profiles of these samples (untreated and AH treated alga) (Figure 5.4) presented similar behavior, with three stages in the degradation pathway. The first stage (< 215 °C) basically corresponds to dehydration of the sample; the second one (215-490 °C) consists in the devolatilization of the sample, and the third stage (> 470 °C) corresponds to the decomposition region, with the remaining mass at the end of this stage being correspondent to the ash (mineral) content in the sample. High mineral content (~26% w/w) was present in the untreated alga, and a considerable amount of this fraction (~17% w/w) remained in the alga after the AH process. These results allow concluding that the AH process was more selective for the extraction of fucoidan than minerals from *F. vesiculosus*.

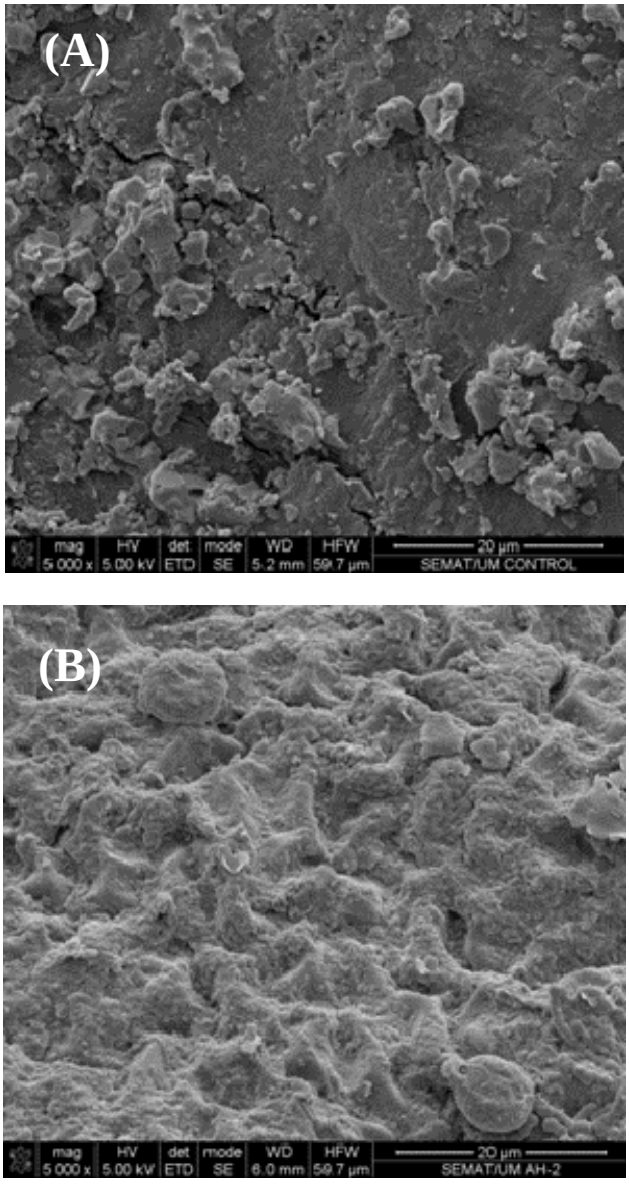

Figure 5.3. Scanning electron micrographs of *Fucus vesiculosus*: (A) untreated sample; (B) residual sample obtained after autohydrolysis at 180 °C for 20. Magnification: 5000-fold.

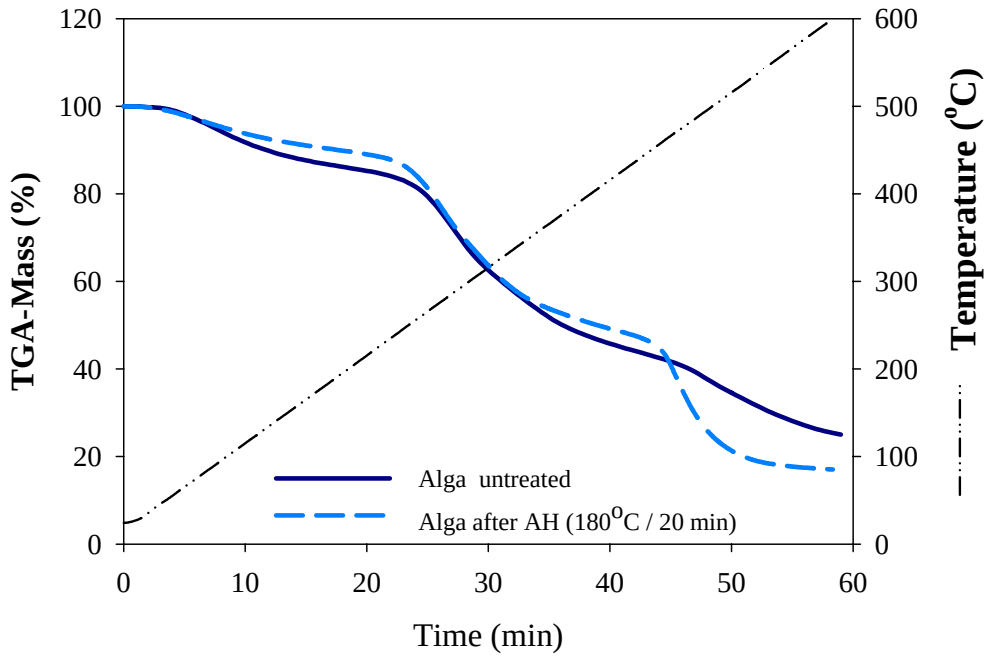

Figure 5.4. Thermal gravimetric analysis (TGA) of *Fucus vesiculosus* before and after the autohydrolysis process under optimized reaction conditions (180 ºC, 20 min).

After optimized the AH conditions, a sequential extraction process was evaluated as a strategy to maximize the fucoidan yield. By using this sequential process of AH, low fucoidan yield (4.47% w/w) was obtained in the second extraction step. This strategy was then considered unviable, since the involved energy costs would not justify the little increase in the extraction yield.

5.3.3 CHARACTERIZATION OF THE EXTRACTED FUCOIDANS

5.3.3.1 Chemical composition

Chemical composition of fucans significantly varies according to the specie, region, and growing conditions of the algal source. Fucoidan obtained from different sources may present different characteristics, and for this reason, elucidating the structure of the

fucoidan sample is very important in order to understand its biological activities. The fucoidan samples obtained in the current study presented a heterogeneous structure mainly composed of fucose and minor proportions of galactose (Table 5.3). In most of the cases, the concentration of fucose was higher than 75% mol. Presence of xylose and glucose was not verified in any sample.

High sulfate content (> 20%) was found in practically all the recovered fucoidans (Table 5.3). This is an advantageous aspect since sulfate groups have been reported to present important biological functions such as anti-HIV activity; and such activity is potentially increased when the sulfate content is increased (Schaeffer and Krylov, 2000). Additionally, all the samples presented a ratio between total sugars and sulfate content (TS/SO_3) higher than 1/0.5 mg TS/mg SO_3, and small quantities of proteins and phenolic compounds (Table 5.3). As a whole, chemical composition of the recovered fucoidan samples is comparable to other reports of sulfated fucans obtained from different sources using different extraction procedures (Anastyuk *et al.*, 2010; Rioux *et al.*, 2007; Rodriguez-Jasso *et al.*, 2011; Wang *et al.*, 2009).

5.3.3.2 FTIR analysis

FTIR spectra of the fucoidan recovered under the optimized AH conditions, as well as other sulfated fucans samples extracted by AH (Figure 5.5) showed typical absorption bands of fucoidans. IR bands at 1200 – 970 cm^{-1} are mainly caused by the C-C and C-O stretching vibrations in the pyranoid ring and C-O-C stretching of the glycosidic bonds. Intense absorption in this region is common for polysaccharides. However, the absorption band at 1240–1255 cm^{-1} (S=O stretching) confirm the presence of sulfate in the recovered polysaccharides. The band at 840 cm^{-1} suggests a complex pattern of substitution of α-linked L-fucopyranose at the axial C-4 position, whereas those at 833-820 cm^{-1} are associated to low amounts of substitution at the equatorial C-2 and C-3 position. (Marais and Joseleau, 2001; Wang *et al.*, 2010).

Table 5.3. Chemical composition of fucoidan samples recovered by autohydrolysis of *Fucus vesiculosus* under different operational conditions. Monosaccharide amount are expressed as the percent of total sugar content in the sample.

Run	Autohydrolysis condition		Fucoidan composition					
	Temperature (°C)	Time (min)	Fucose (% mol)	Galactose (% mol)	SO_3 (%)	TS:SO_3 [a] (mol:mol)	Protein (mg L^{-1})	Phenols [b] (%)
1	200	30	52.21	47.79	30.78 ± 2.13	1 / 1.82	5.55 ± 1.03	3.15 ± 0.53
2	200	10	83.96	16.04	21.02 ± 2.33	1 / 0.96	5.11 ± 0.83	3.69 ± 0.16
3	160	10	81.16	18.84	23.67 ± 1.40	1 / 1.33	4.97 ± 0.17	4.84 ± 0.16
4	160	30	75.08	24.92	25.59 ± 2.42	1 / 1.14	5.51 ± 0.31	5.38 ± 0.06
5	180	10	74.12	25.88	22.08 ± 1.16	1 / 0.95	10.39 ± 2.13	3.95 ± 0.21
6	180	30	55.14	44.86	20.96 ± 2.51	1 / 0.79	10.90 ± 1.00	4.87 ± 0.46
7	200	20	51.79	48.21	19.06 ± 0.44	1 / 1.00	12.41 ± 0.36	3.99 ± 0.91
8	160	20	77.75	22.25	18.46 ± 0.66	1 / 1.08	9.21 ± 1.37	3.35 ± 0.01
9-12 [c]	180	20	76.76	23.24	21.21 ± 0.76	1 / 0.87	8.56 ± 0.57	5.63 ± 0.78

[a] TS/SO_3 = (mg TS/100 mg fucoidan)/(mg SO_3/100 mg fucoidan). TS: total sugars

[b] mg of total phenols/100 mg of fucoidan

[c] Mean value of 4 centre point assays

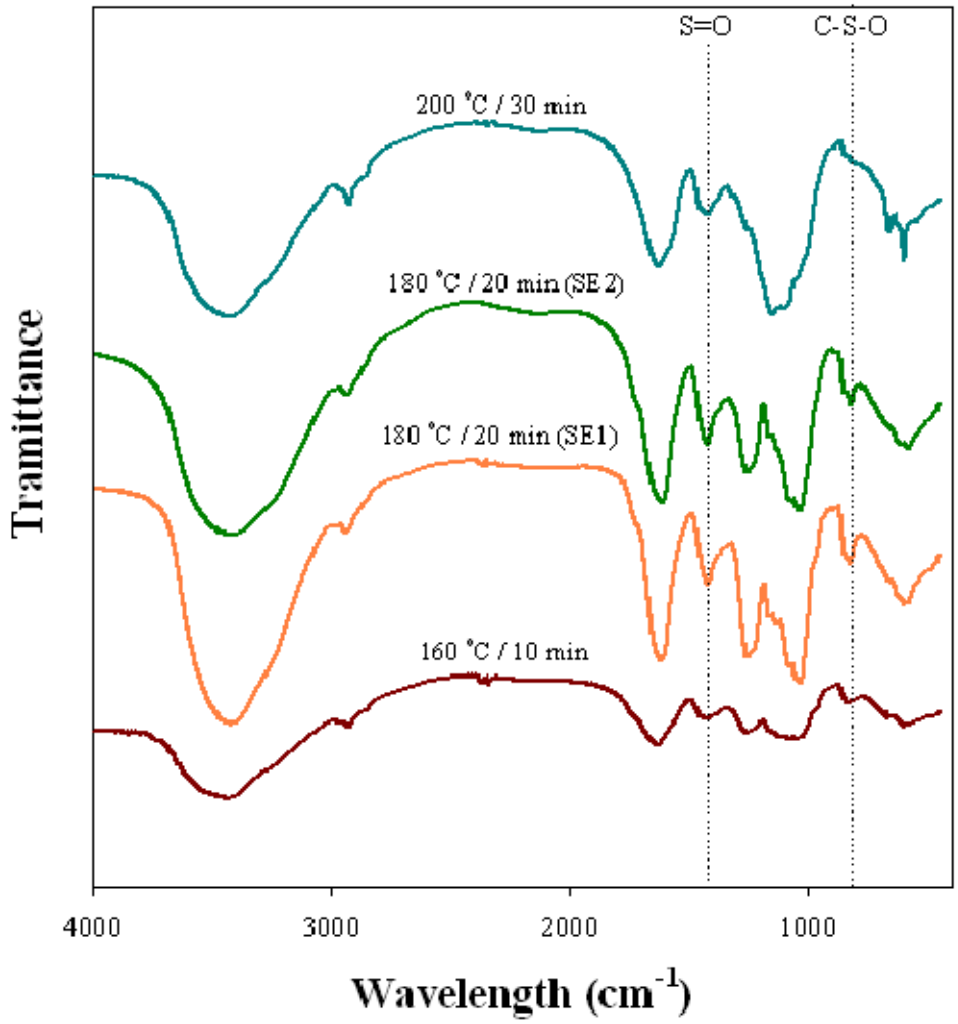

Figure 5.5. Infrared analysis spectroscopy (FTIR) of fucoidan samples obtained by autohydrolysis of *Fucus vesiculosus* under different operational conditions.

5.3.3.3 Thermal analysis

TGA and DSC curves of fucoidan extracted under the optimized AH conditions are shown in Figure 5.6. Similar profile for sulfated fucans is reported by Rodriguez-Jasso *et al.*, (2011); however, in the present study the volatile matter evolution for AH-fucoidan presented a constant decrease of mass without showing the stationary stage in the decomposition region. This fact suggests that the use of higher temperature (>600 ºC) would be required to reach this stage. In any way, it is possible to verify that

the fucoidan sample contained low quantity of mineral compounds (sulfates, phosphates and carbonates).

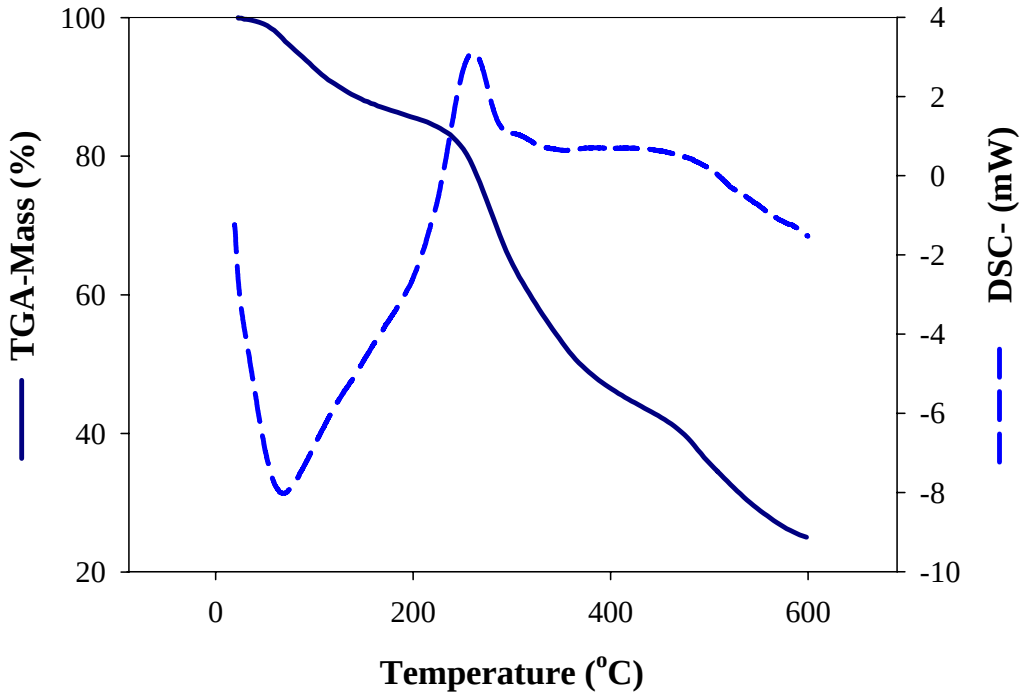

Figure 5.6. Thermal gravimetric analysis (TGA) and differential scanning calorimetry (DSC) of the fucoidan sample recovered under the optimized autohydrolysis conditions (180 ºC, 20 min).

5.4 CONCLUSIONS

Autohydrolysis process was a suitable technology for extraction of sulfated polysaccharides from *Fucus vesiculosus*. The use of this technology brings important advantages from economical and environmental viewpoints since when compared to conventional extraction procedures, the autohydrolysis method is more environmentally friendly (it does not require the use of chemical solvent and generates less waste). The current study revealed that the fucoidan yield, as well as the fucose and sulfate contents in the polysaccharide were affected by the temperature and reaction time used for autohydrolysis. Optimization of the extraction conditions was a useful strategy to maximize the fucoidan yield (~16.5% w/w).

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CHAPTER 6

ANTIOXIDANT ACTIVITY OF SULFATED FUCANS EXTRACTED BY HYDROTHERMAL PROCESSES

ABSTRACT

The antioxidant activity of sulfated fucans obtained by microwave-assisted extraction and autohydrolysis of *Fucus vesiculosus* seaweed was determined. Five different methods were used to estimate the antioxidant activity, including free radical-scavenging (ABTS and DPPH), lipid oxidation (LOI), ferric reducing capacity (FRAP), and differential pulse voltammetry (DPV). Results showed a strong antioxidant activity in both sulfated fucans, with higher effect over $ABTS^{\cdot+}$ radicals' inhibition. Such fucans could serve as free-radical inhibitors or scavengers, acting possibly as primary antioxidants.

6.1 INTRODUCTION

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative and chronic diseases including alcoholic liver disease, aging, and diabetes. Reactive oxygen species (ROS) such as superoxide anions ($O2^{-\bullet}$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) are constantly generated by normal metabolic processes or from exogenous factors and agents. The excessive production of ROS may result in oxidative damage to many large biomolecules, such as lipids, DNA, and proteins, leading to destruction of the cellular membrane or tissue damage. (Sokolova *et al.*, 2011; Wang *et al.* 2010). Additionally, lipid peroxidation is a major cause of many pathological effects such as cardiovascular disease, cancer, and brain dysfunction; also leading to the development of food rancidity and off-flavors (Lim *et al.*, 2002).

Recently, the search for natural substances with multifunctional properties that can delay or prevent oxidation of cellular substrates has drawn more and more attention in order to avoid the use of synthetic compounds related to toxic and carcinogenic effects on health. Therefore, since the ocean is considered to be a source of potential drugs, the major occupation of marine natural products chemists has been the search for novel bioactive metabolites for prospective pharmaceuticals and healthy foods. In 2005, Bhakuni and Rawat, reported that approximately 16,000 natural products have already been isolated from marine organisms, which were described in approximately 6,800 publications. Several of these compounds exhibit potential for application in medical, pharmaceutical, cosmetic and food industries (Bhakuni and Rawat, 2005). Among marine species, seaweeds are known to be able to generate bioactive compounds to protect themselves from external factors such as UV radiation, stress and herbivores. Researchers have also revealed that compounds derived from marine algal material exhibit various biological and physiological activities (Wijesekara and Kim, 2011). As a consequence their value as source of novel bioactive substances has grown rapidly.

Brown seaweed sulfated polysaccharides (fucans), have been reported as polydisperse molecules composed of variable amounts of fucose, galactose, xylose, and sulfate, with significant economical importance due to their extensive use in biochemical and

biotechnology. Several studies have demonstrated their properties as free-radical scavengers and antioxidants for the prevention of oxidative damage in living organisms (Ruperez *et al.*, 2002; Bhakuni and Rawat, 2005; Costa *et al.*, 2010). Moreover, fucans as antioxidants has been classified as non toxic and water-soluble macromolecules, playing an important role in assisting their water insoluble counterparts in the removal of reactive oxygen species from the body (Ruperez *et al.*, 2002; Sokolova *et al.*, 2011). However, fucans structure and biological properties including antioxidant capacities varies according to algal species, environmental conditions, life stage of seaweed, and extraction procedure (Costa *et al.*, 2010).

Hydrothermal processes have been considered an environmentally friendly and cost-effective technologies that allow biomass fractionation into its main constituents using only water as the only reactant under high temperature and pressure. Some of the main advantages of these processes include the use of water and no other chemical reactive, limited equipment corrosion problems, simple and economical operation (Pronyk *et al.*, 2011; Ruiz *et al.*, 2011).

In our previous works, the extraction conditions and structural characterization of *Fucus vesiculosus* fucoidan using two hydrothermal methods (microwave and autohydrolysis) were studied (Rodriguez-Jasso *et al.*, 2011a-b). The highest yields were obtained using low residence time and extraction temperatures between 170-180 °C. Due to the known biological activities of sulfated fucans, it was presumed that the obtained fractions by microwave and autohydrolysis processes could possibly exhibit differences between its biological properties. For that reason, the aim of this chapter was to compare ABTS, DPPH, FRAP and DPV assays to estimate the antioxidant activities of sulphated fucans extracted by hydrothermal processes.

6.2 EXPERIMENTAL PROCEDURES

6.2.1 VEGETAL MATERIAL AND CHEMICALS

Fucus vesiculosus seaweed was collected from the Praia Norte, Viana do Castelo, Portugal during September 2009 and May 2010 (microwave and autohydrolysis extraction, respectively). 1,1-Diphenyl-2-picryl-hydrazyl (DPPH$^{\bullet}$), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet+}$), ferrous sulfate and iron (III) chloride, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and linoleic acid, 95% were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Anthrone reagent was from Prolabo, Normapur. Coomassie Plus (Bradford) Assay Kit was from Thermo Scientific Co. All other reagents used were of analytical grade. Hydrothermal process

6.2.2 SULFATED FUCANS ISOLATION

Before extraction, the fresh seaweed was washed to remove sand and epiphytes, dried at 35 ºC, and milled in a home blender to a size higher than 1000 μm. The conditions used for microwave and autohydrolysis extraction of sulfated fucans isolation were previously established, and consisted in using an alga/water ratio of 1/25 (w/v), at 172°C for 1 min, and 180 °C for 20 min, for microwave and autohydrolysis systems, respectively (Rodriguez-Jasso *et al.*, 2011a-b).

6.2.3 ANALYTICAL DETERMINATIONS

Total sugar content was determined by the anthrone method; and total phenolic compounds by the Folin-Ciocalteau assay. The content of sulfate groups was quantified through the barium chloride–gelatin method (Dodgson, 1961). All absorbance measurements were performed in triplicate.

6.2.4 ANTIOXIDANT ACTIVITY ASSAYS

To determine the antioxidant activity of the sulfated fucans, solutions were prepared by solubilizing the polysaccharides in distilled water to 500 and 1000 ppm. The total antioxidant activity was then estimated by the methods described below:

6.2.4.1 DPPH$^{\bullet}$ scavenging capacity assay

The scavenging activity of the DPPH$^{\bullet}$ free radical was assayed according to the methodology proposed by (Randhir and Shetty (2007). Briefly, a 60 µM solution of DPPH was prepared, and 2,950 µL of this solution was added to 50 µL of sample.The mixture was vigorously shaken and incubated for 30 min in the dark at room temperature. The reduction of the DPPH$^{\bullet}$ radical was measured by continuous monitoring of the decrease of absorption at 517 nm. The control solution contained 100 µL of distilled water. DPPH$^{\bullet}$ percent of inhibition was calculated by the following equation:

$$Inhibition\ (\%) = \left[\left(1 - A_{sample\_517}/A_{comtrol\_517}\right)\right] * 100$$

The radical-scavenging capacity of each sample was calculated according to Trolox standard curve (0 to 100 ppm) and expressed as Trolox equivalent antioxidant capacity (TEAC) by the extrapolation of DPPH• percent of inhibition of each tested sample.

6.2.4.2 ABTS$^{\bullet+}$ scavenging capacity assay.

ABTS$^{\bullet+}$ assay was carried out according to the methodology described by (Martínez-Avila *et al*. (2012). For radical (ABTS$^{\bullet+}$) formation, 12.5 mL of potassium persulfate 2.45 mM were mixed with 25 mL of ABTS 7 mM solution; and the mixture was maintained in the dark at room temperature for 12-16 h before use. Then, the absorbance was measured at 734 nm, and the ABTS$^{\bullet+}$ solution was diluted with ethanol until obtain an absorbance value of 0.7 ± 0.01. For the assays, 950 µL of ABTS$^{\bullet+}$ solution were added to 50 µL of sample, and the absorbance was measured after one

minute of reaction. The control solution contained 50 µL of distilled water instead of sample. $ABTS^{\bullet+}$ percent of inhibition was calculated by the following equation:

$$Inhibition\ (\%) = \left[\left(1 - A_{sample\_734}/A_{comtrol\_734}\right)\right] * 100$$

The radical-scavenging capacity of each sample was calculated according to Trolox standard curve (0 to 100 ppm) and expressed as Trolox equivalent antioxidant capacity (TEAC) by the extrapolation of $ABTS^{\bullet+}$ percent of inhibition of each tested sample.

6.2.4.3 Ferric reducing antioxidant potential (FRAP) assay

The FRAP assay was done according to Benzie and Strain (1996) with some modifications. Sulfated fucans samples were prepared by mixing 10 µl of sample with 290 mL of FRAP reagent in a 96-well microplate. Then, the reaction mixture was incubated at 37 °C for 15 min. After that, the absorbance was determined at 593 nm against a blank prepared using distilled water. The working FRAP reagent was daily prepared by mixing a 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl with a 20 mM FeCl3 solution and 0.3 M acetate buffer (pH 3.6) in a proportion 1:1:10 (v/v/v). Standard calibration curve was prepared using FeSO4.7H2O (range 200 - 1000 µM).

6.2.4.4 Lipid oxidation inhibition (LOI) assay

The lipid oxidation inhibition (LOI) was performed using linoleic acid as lipid source according to the method described by Starzyńska-Janiszewska *et al.*, (2008) with slight modifications. Substrate solution was prepared by diluting 0.56 g of linoleic acid and 1.5 g of Tween 20 in 8 mL of ethanol at 96 %. Each solution of sulfated polysaccharides (50 µL) was mixed with 100 µL of linoleic acid solution and 1.5 mL of 0.02 M acetate buffer pH 4.0. The controls solution contained 50 µL of distilled water. All the samples were homogenized in a vortex and the emulsions were incubated at 37 ºC for 1 min. Then, 750 µL of 50 M $FeCl_2$ solution (0.0994 g $FeCl_2$ and 0.168 g EDTA diluted to 1 L with distilled water) were added in order to induce the oxidation of linoleic acid. After

1h, 1 mL of 0.1 M NaOH in 10 % ethanol was added to 250 µL of the mixture to stop the oxidation process. After mixing, 2.5 mL of 10 % ethanol was added and the absorbance measured at 232 nm against 10 % ethanol as blank. The percent of antioxidant activity was calculated according to the following equation:

$$\text{Lipid oxidation inhibition}(\%) = 100 - (B \cdot 100 - A)$$

where A is the difference between the absorbance of control sample (distilled water) after 24 h and initial time of incubation (0 h), and B is the difference between the absorbance of the sample after 24 h and initial time h of incubation.

6.2.4.5 Differential pulse voltammetry (DPV)

The principle of this method involves the recording of voltammograms of the cathodic reduction of oxygen by means of any voltammetric analyzer using differential voltammetry. Assays were performed on a Potentiostat/Galvanostat BASi-Epsilon equipment using a closed standard three electrode cell. A Pt foil electrode was used as a working electrode and a Pt spiral electrode as a counter. All potentials were refer to an Ag/AgCl 3 M KCl reference electrode. The height of the pulses was adjusted to 80 mV with a potential range from 0 to 1700 mV. Pulse amplitude and width were 200 and 50 ms, respectively and the clock time fixed to 2 s. was 200 ms, pulse width the current was sampled during the final 17 ms of the pulse. The electrochemical evaluation of sulfated fucans antioxidant potential was compared against a standard solution of Trolox (1000 ppm).

6.2.5 STATISTICAL ANALYSIS

Statistical analyses were performed using analysis of variance (ANOVA). The multiple comparison test (α = 0.05) was used to determine any significance of differences between specific means (Matlab, version 7.8.0, R2009a software, USA).

6.3 RESULTS & DISCUSSION

6.3.1 CHEMICAL CHARACTERIZATION

Table 6.1 shows the chemical characterization of the sulfated fucans used in the present study. The selected polysaccharides were obtained after carried out a group of experiments previously reported in Chapter 4 and 5, where the highest yields of microwave (MSF) and autohydrolysis (ASF) sulfated fucans, were 18.22 and 16.48% (dry weight), respectively. L-fucose was the main monosaccharide in both samples, however significant differences between these fucans were observed according to the hydrothermal treatment employed. Therefore, the heterogeneous and branched structure of fucoidan was highly affected by the autohydrolysis process, which could be related to the longer reaction time used when compared to the microwave-assisted extraction. On the other hand, similar sulfate content was found in both samples (> 20% w/w). High sulfate degree on fucans enhances the polysaccharide water solubility, which can result in the alteration of important biological functions (Yang *et al.*, 2011). The polyphenols contents obtained by both hydrothermal methods showed a low content with values of approximately 5% (w/w). This low values can be associated with the use of water as reagent, since is known that phenolic compounds are generally more soluble in polar organic solvents than in water (Aguilar *et al.*, 2008).

Table 6.1. Chemical characterization of the sulfated fucans obtained by microwave and autohydrolysis processes.

Sample	%Fucoidan	Fucose (%mol)	$\%SO_3$	mgTS /100 mg Fuc	Phenols
MSF	18.22 ± 1.40	53.80 ± 1.19	21.09 ± 1.65	27.26 ± 1.50*	5.01 ± 0.60
ASF	16.48 ± 1.21	76.76 ± 2.32*	21.21 ± 1.12	24.38 ± 2.24	5.63 ± 0.79

6.3.2 ANTIOXIDANT ACTIVITY OF THE SULFATED FUCANS

The antioxidant capacities of free radical scavenging, ferric reducing ability and lipid oxidation of the extracted fucans was evaluated by solubilizing the samples in two different concentrations. As can be observed in Figure 6.1 all the evaluated assays showed strong antioxidant activities with the increase of fucoidan concentration of both the microwave and autohydrolysis extract, demonstrated the inhibition of free radical generation are with the increase of the concentrations.

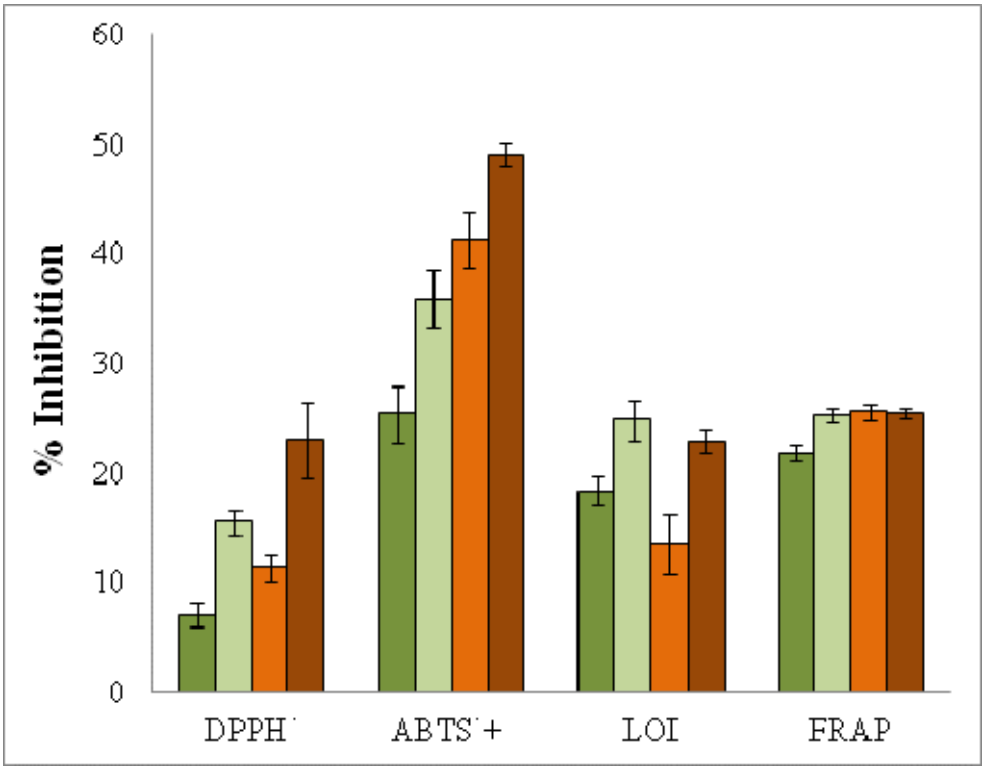

Figure 6.1. Inhibitory effect of DPPH$^{\bullet}$, ABTS$^{\bullet+}$, LOI and FRAP antioxidant capacity of microwave (MSF) and autohydrolysis (ASF) sulfated polysaccharides.
MSF-500 ppm; MSF-1000 ppm; ASF-500 ppm; ASF-1000 ppm; Trolox.

Radical scavenging capacities were determined using DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays. The DPPH$^{\bullet}$ free radical scavenging model has been widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants. The absorbance decreases as a result of a color change as the radical is scavenged by antioxidants through the donation of hydrogen to form the stable DPPH–H molecule (Hu *et al.*, 2004). In the present

study, the highest $DPPH^{\bullet}$ radical scavenging effects were observed for the fucoidan sample obtained by autohydrolysis, with an inhibition value of 22.98% ± 3.49 at a concentration of 1 mg mL^{-1}, which was 50% higher than the inhibition observed for the fucoidan obtained by microwave-assisted extraction.

In previous studies carried out by Zhang *et al* (2011), the scavenging effect of butylated hydroxyanisole (BHA), at the concentration mg mL^{-1}, was only 12.2%, hence the obtained values for both extracted analysis showed higher scavenging activities for $DPPH^{\bullet}$ radicals compared BHA, presuming that sulphate groups in fucans molecule can activate the hydrogen atom of the anomeric carbon; and its indicated that MSF and ASF showed a strong proton-donating ability and can serve as free-radical inhibitors or scavengers.

In order to compare the antioxidant activity of the extracted polysaccharides we analyzed the change in the $ABTS^{\bullet+}$ absorbance after 1 min of reaction was analyzed. At this time, it can be considered that the fast reaction is completed, and the contribution of the slow reaction to the decrease in absorbance of the cation radical is little. The results obtained with Trolox as reference showed that the $ABTS^{\bullet+}$ radical values ranged from 25 to 50% of inhibition, which represents a higher variation than in the $DPPH^{\bullet}$ assay correspondent to approximately 2.32 and 2.13-fold for microwave sulfated fucan (MSF) and authohydrolysis fucan (ASF) at 1 mg mL^{-1}, respectively. As observed with the $DPPH^{\bullet}$ assay, the highest $ABTS^{\bullet+}$ radical values were obtained for the autohydrolysis extracts, with an antioxidant capacity of 49.06 ± 1.05. Barahona *et al.* (2011) obtained higher antioxidant capacity values, measured by the $ABTS^{\bullet+}$ radical cation, for *Lessonia vadosa* fucoidan compared against carrageenans, implying that the scavenging effect of fucoidan may due to the sulfate group position at O-2, close to the glycosidic bond. Their finding suggested that the chemical structure of fucans plays some role on the H abstraction reaction by the $ABTS^{\bullet+}$ cation radical.

Lipid oxidation technique (LOI) was studied as complimentary analysis to the data obtained by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ analyses, with the purpose to provide more precise information about the antioxidant properties in real biological systems. Therefore, the method simulates the lipids oxidization conditions using linoleic acid as a lipid source (Martínez-Avila *et al.*, 2012). As can be seen in Figure 6.1, both fucoidan samples

showed stronger LOI effect at 1 mg mL^{-1}, with a scavenging effects over to 22%. On the contrary to the previously mentioned antioxidant assays, MSF showed the highest percentage of lipid inhibition.

The FRAP assay as a reducing capacity serves as a significant indicator for potential antioxidant activity. The reductants (antioxidants) can result in the reduction of the Fe 3+ /ferricyanide complex to the ferrous form (Fe 2+), while the color of test solution is changed with the reducing power of the antioxidant samples (Zhao *et al.*, 2006). The reducing powers of MSF and ASF by FRAP assay showed that the trend for the ferric ion reducing activities did not vary markedly among the extraction method and concentration of the solution, being obtained reducing capacity values higher than 25% for all the samples (Figure 6.1). The reducing properties are generally associated with the presence of reductant, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductant are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Zhang *et al.*, 2010). Hence, the ASF and MSF reducing properties may be related to sulphonic group that can as electron donors and can react with free radicals to form more stable products.

Based on the fact that the antioxidant compounds can act as reduction agents and, in solutions, they tend to be easily oxidized at inert electrodes, alternative methods have established an interesting relationship between electrochemical behaviour of the antioxidant compounds and their resultant 'antioxidant power" (Barros *et al.*, 2008). Differential pulse voltammetry (DPV) is a simple technique that involves the current recording of the electrochemical oxygen reduction at the electrode surface in order to generated the data necessary to calculate the antioxidant capacity (Korotkova *et al.*, 2003). The voltamperograms generated after DPV with MSF and ASF are showed in Figure 6.2, where the values of intensities and potentials of sulfated polysaccharides are registered with the peaks signal formation and the change of these signals with the samples concentration. A remarkable difference was observed in the oxidation process between the Trolox solution, used as standards, and the sulfated fucans samples. The peak´s maximum current where recorded at 0.49 V, with and intensity of 0.02 and 0.064 mA for MSF and ASF extracts, respectively, at 1 mg mL^{-1}. The intensity value

increased up to more than 32 times, when recording a DPV with the ASF sample compared against MSF extract. Hence, ASF can be defined with stronger antioxidant activity because the intensity value obtained by DPV is directly related with antioxidant activity power, and it can be used as a rapid index to evaluate the biological properties of bioactive molecules.

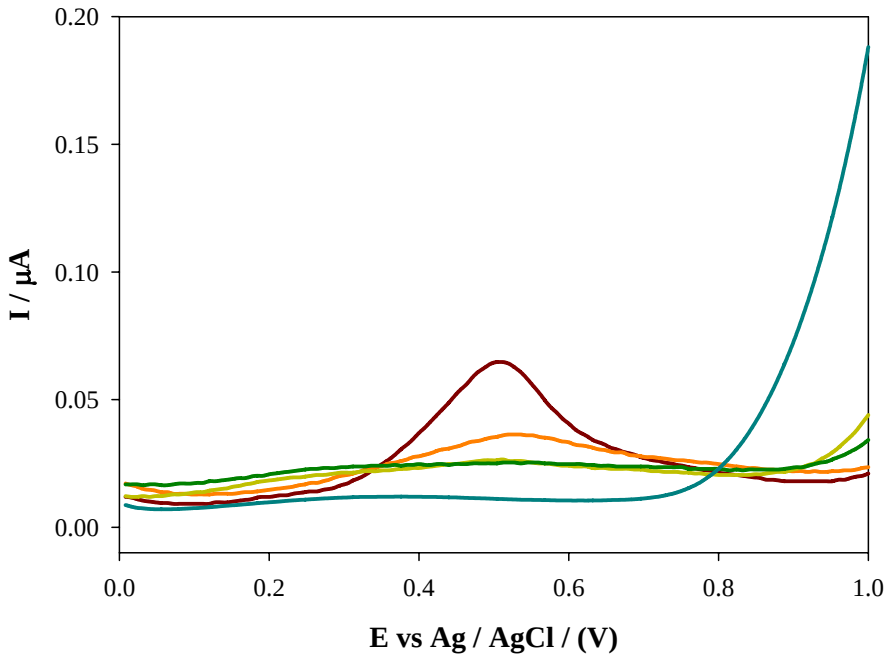

Figure 6.2. Differential pulse voltamogram of 500 and 1000 mg mL^{-1} of microwave (MSF) and autohydrolysis (ASF) sulfated polysaccharides. MSF-500 ppm (▬); MSF-1000 ppm(▬); ASF-500 ppm (▬); ASF-1000 ppm (▬); Trolox (▬).

6.3.3 COMPARATIVE ANALYSIS AMONG DPPH•, ABTS•+, LOI AND FRAP ASSAYS

The antioxidant activity results obtained for the sulfated fucans, measured by the DPPH•, ABTS•+, LOI and FRAP techniques (using a sulfated fucan solution of 1 mg mL-1), were statistically compared. The ANOVA test showed that antioxidant capacities were significantly different among the assays ($p < 0.05$). FRAP and LOI results were not different among antioxidant assays and extraction procedures (Table 6.2). DPPH• assay showed the lowest significant effect for MSF samples. Meanwhile,

the highest effects were observed for ABTS•+ with autohydrolysis and microwave fucoidan, which showed significant differences when compared to all the antioxidant assays and extractive procedures. However there is also observed significant difference among this hydrothermal processes, with highest antioxidant activity in autohydrolysis sulfated extracts (letter F). Therefore, ABTS•+ could be considered an appropriate technique to determine the antioxidant activity of sulfated fucans.

Table 6.2. Significant differences of antioxidant activity mean values of MSF and SMF extracts.

Microwave (MSF)				Autohydrolysis (ASF)			
DPPH$^{\cdot}$	ABTS$^{\cdot+}$	LOI	FRAP	DPPH$^{\cdot}$	ABTS$^{\cdot+}$	LOI	FRAP
A							
	B						
		C	C	C		C	C
		D	D	D		D	D
		E	E	E		E	E
					F		
		G	G	G		G	G
		H	H	H		H	H

The letters A-H represent the statistical similarity among data from the same rows

6.4 CONCLUSIONS

Microwave and autohydrolysis sulfated polysaccharides exhibited obvious and comparable antioxidant activities with DPPH$^{\bullet}$, ABTS$^{\bullet+}$, LOI and FRAP assays. The highest activity was obtained for autohydrolysis sulfated extracts, especially with the ABTS$^{\bullet+}$. This behavior was confirmed with the oxidation capacity of ASF extracts reported by the strong intensity peak obtained by DPV assay. Results indicated that the presence of sulphate groups can increase the scavenging activity, lipid oxidation and the binding of the metal ion, however further studies are needed to explain the differences obtained after the different hydrothermal extractions specifically in the relation between the antioxidant activity and their mechanisms with the polysaccharides structure

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CHAPTER 7

FUNGAL FUCOIDANASE PRODUCTION IN A SOLID STATE ROTATING DRUM BIOREACTOR USING ALGAE BIOMASS AS SUBSTRATE

ABSTRACT

Specific glycosyl hydrolase enzymes able to degrade fucoidan were produced by solid state fermentation using a laboratory-scale rotating drum bioreactor. Two fungal strains and three algae substrates were evaluated; also, rotational and stationary fermentations were carried out in order to determine the mixture effect over the solid support. The conditions that promoted the maximum fucoidanase activity were then used in a larger scale rotating drum bioreactor. In this step, the effect of the substrate moisture content on the enzyme production was investigated. Assays combining the algae substrate with an inert support (synthetic fiber) were also carried out. Agitated experiments showed advantages in the induction of enzyme activity when compared to the static ones. Fermentation of the autohydrolyzed alga by *Mucor* sp. 3P gave the highest enzyme activity.

7.1 INTRODUCTION

Fucoidans are marine hetero-polysaccharides with a wide spectrum of activity in biological systems. Besides their well-attested anticoagulant and antithrombotic activity, they act on the inflammation and immune systems, have antiproliferative and antiadhesive effect on cells, protect cells from viral infection, and can interfere with mechanisms involved in fertilization (Berteau and Mulloy, 2003; Giordano *et al.*, 2006)

Enzymes with known specificities that catalyze the degradation of fucoidans (fucoidan hydrolase, fucosidase) are important tools for studying the structural peculiarities and the biological role of this class of polysaccharide. Fucoidanase may cleave glycosidic bonds in the core of the polysaccharide, leading to a rapid reduction of the molecular weight (endo-fucoidanase), or in the edge of the polysaccharide, releasing some oligosaccharides with little decrease of the molecular weight (exo-fucoidanase) (Furukawa *et al.*, 1992). Fucoidanase enzymes have been found in many invertebrates and marine microorganisms; however most of them exhibit weak titles of activity (Bakunina *et al.*, 2000; Burtseva *et al.*, 2000; Alexeeva *et al.*, 2002)

Solid state fermentation (SSF) is defined as the growth of microorganisms on a solid matrix with a low content of free water. SSF has been widely used in recent years due to promote high productivity of several bioactive compounds (secondary metabolites) principally with agro-industrial residues, increasing the interest on their applications (Aguilar *et al.*, 2008a). Additionally, the reutilization of agro-industrial wastes for enzymes production by SSF minimize the pollution and allows obtaining these high added-value products using an economical technology (Aguilar *et al.*, 2008b; Singhania *et al.*, 2009). When compared to the liquid media used in submerged fermentation systems, the solid media used in SSF contain less water, but they present an important gas phase between the particles (Durand, 2003). This condition favors the development of filamentous fungi, which have great capacity to colonize the interparticle spaces of solid matrices.

Many bioreactors have been traditionally used in SSF processes; mainly classified as the possibility to work as an agitated system or in static conditions. The agitated

systems are principally comprises for rotating drums, gas-solid fluidized beds, rocking drums, horizontal paddle mixer, etc, while the static ones includes the packed-bed and the trays bioreactor (Durand, 2003; Ruiz-Leza *et al.*, 2007). On the other hand, the use of mixed bioreactors improves the homogeneity of the bed and ensures an effective nutrients transfer.

This study aimed to evaluate the production of fucoidan hydrolytic enzymes by fungal strains through SSF in a laboratory-scale rotating drum bioreactor, using different pretreated alga as substrates. The scaling up of this system on the enzyme production was also investigated.

7.2 EXPERIMENTAL PROCEDURES

7.2.1 MICROORGANISM AND INOCULA

Aspergillus niger PSH and *Mucor sp.* 3P strains belonging to the DIA/UAdeC fungal collection (Food Reaserch Department/University of Coahuila, Mexico), conserved under cryopreservation conditions (- 80°C) were used in this study. Potato dextrose agar (PDA-Difco) plates were applied for spores propagation at 27 °C. To be inoculated in the fermentation media, spores from 7 days sporulated cultures were collected in 0.1% (w/v) Tween 80 solution and counted in a Neubauer chamber to be inoculated in the fermentation media.

7.2.2 RAW MATERIALS

Microwave and autohydrolysis processed algae biomass from *Fucus vesiculosus* specie (collected from the Praia Norte, Viana do Castelo, Portugal, during spring 2011), were evaluated as substrate in SSF experiments; untreated alga was also tested as control assay. Microwave and autohydrolysis pre-treatment conditions were based in the results obtained in Chapters 4 and 5 (172 °C for 1 min, and 180 °C for 20 min, respectively). Chemical characterization of the algal materials was carried out by mixing 0.23 g of

algae with 2.5 mL of an 11.6% (v/v) H_2SO_4 solution (Kim et al, 2011). The reaction was maintained at 121 °C for 15 min, being immediately stopped in an ice bath. Hydrolyzed samples were filtrated and the final volume was adjusted to 10 mL by addition of NaOH 1 M (pH 5) for future analysis of total sugar, proteins, total phenols and monosaccharide contents.

7.2.3 INVASION CAPACITY OF FUNGAL STRAINS

The fungal growth was evaluated in Petri dishes. For the experiments, untreated alga (A), autohydrolyzed alga (AH) and microwave processed alga (AM) biomass were used as sole carbon source, and their moisture content was adjusted to 80% by addition of Czapek-Dox modified medium composed of (g L^{-1}): Urea (5.0); KH_2PO_4 (1.5); KCl (0.5); $MgSO_4$ (0.5); Hunter traces (0.1) (pH 5.0) (Hill and Kafer). Each strain was inoculated by puncture in the center of 60-mm Petri dishes with a suspension containing 1×10^7 spores mL^{-1}. Radial growth was monitored kinetically, every 4 h during the day and 8 h during the night, using a digital micrometer (Mitutoyo 293-561, Japan) (Rodriguez-Jasso *et al.*, 2010). The growth curves data corresponding to the exponential phase were analyzed by linear and exponential regressions to estimate biokinetics variables as radial growth rate (*Ur*, mm h^{-1}), and duplicity time (t_d, h) (Vonshak and Maske, 1985; Loera and Viniegra, 1998).

A scanning electron microscope (Nova NanoSEM 200, Netherlands) was used to visualize the mycelia growth on the different morphology of the algae substrates. Samples were coated with layer of gold by sputtering with an accelerating voltage varying to 15 kV.

7.2.4 ROTATING DRUM BIOREACTOR AND FERMENTATION CONDITIONS

The designed and constructed laboratory-scale rotating drum bioreactor consisted of a horizontal module with six independent acrylic columns (36 cm height x 6 cm diameter), each one connected to a 10 rpm motor. Each column supported three 50-mL syringes that contained the solid substrate Figure 7.1.

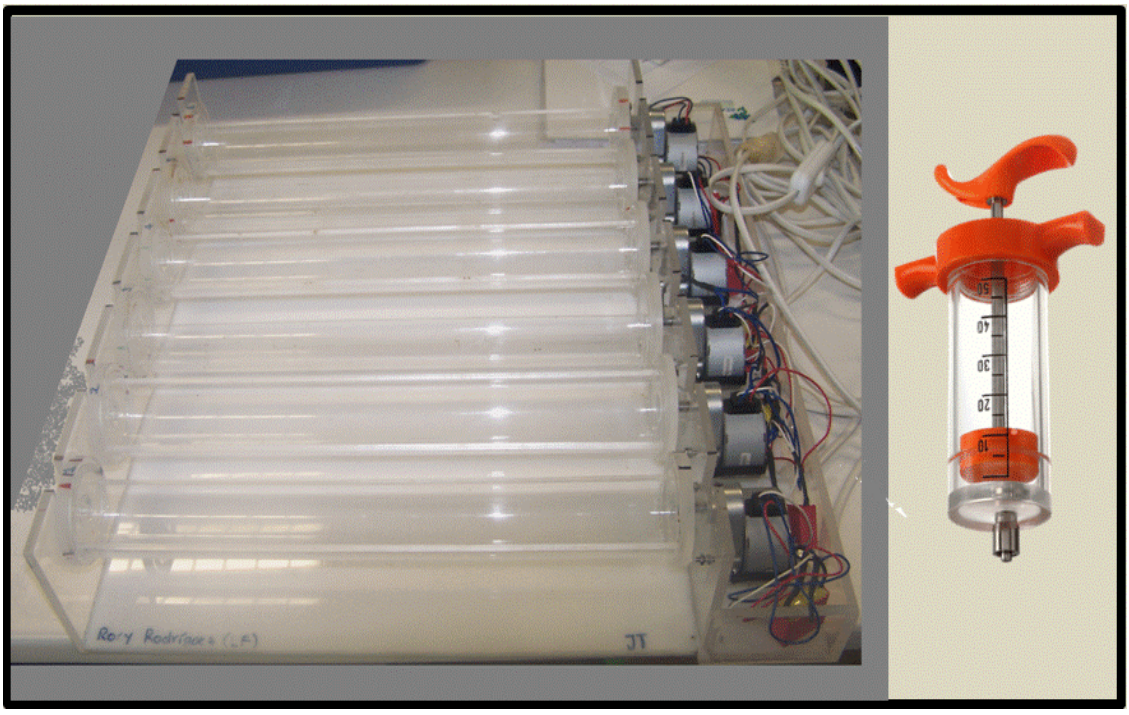


Figure 7.1. Laboratory scale rotating drum bioreactor:
a) serial column module; b) 50 mL syringe.

For the fermentations assays, two fungal strains (*Aspergillus niger* PSH and *Mucor sp* 3P), three algae biomass (untreated, microwave processed, and autohydrolyzed); and two rotation conditions (0 and 10 rpm) were evaluated. Before the SSF experiments, algae biomass was dried at 35 °C and sterilized at 100 °C during 60 min. Each syringe was filled with 4 g of solid substrate pre-inoculated with $1x10^7$ spores g^{-1} of support, moistened with Czapek Dox modified media (pH 5) to achieve 70% moisture content. The system was incubated at 30 °C during 96 h. Samples were withdrawn every 12 h and filtered through nylon membrane in order to recover the fermentative media. All the experiments were done in triplicate.

7.2.5 FERMENTATION CONDITIONS FOR SCALE UP EXPERIMENTS

Autohydrolyzed algae biomass for scale up experiments was produced in an 18-L total volume stainless steel reactor (Parr Instruments Co). Pre-treatment was carried out at

160 °C in non isothermal profile, with an agitation speed set at 120 rpm. At the end of the treatment, the solid phase was separated by filtration and the recovered solids were washed with distilled water. The excess of water in non-isothermal algae biomass (NHA) was eliminated by centrifugation and the moisture content was quantified. The autohydrolysis liquor was filtered and stored at 4 ºC.

In this step of the study, the effect of the type of substrate and culture media used to moisten them, on fucoidanase production was evaluated. Three different algae biomass were used: a) fresh untreated algae (U_fA); b) non-isothermal autohydrolyzed alga (NHA) and c) non-isothermal autohydrolyzed alga supported on synthetic fiber cubes (Green Scotch Brite, 4×4 mm (SHA). The moisture content of the solid substrates were adjusted and maintained at 80% during SSF experiments, by adding water or autohydrolysis liquor to algae biomass. Culture media were also supplemented with Czapek dox salts at the same proportions mentioned in the above section.

SSF assays were performed in a rotating drum bioreactor with 500-mL cylinders. Each cylinder contained 50 g of wet substrate (70%) inoculated with spores of *Mucor sp* 3P ($1x10^7$ spores g^{-1} dry support). Moisture content was adjusted to 80% with the correspondent culture media (approximately 25 mL, pH 5). Experiments with ASH also contained 10 g of synthetic fiber cubes, previously washed by boiling in distilled water for 30 min and then drying overnight at 60 °C (Mussatto *et al.,* 2009). The system was maintained at 30 °C during 120 h. All the experiments were carried out in duplicate.

7.2.6 ANALYTICAL METHODS

7.2.6.1 Monosaccharide content in algae biomass

The monosaccharide composition of algae substrates were quantified by HPLC using a Jasco chromatograph system (Jasco, Tokyo, Japan) equipped with a low-pressure gradient solvent pump, an autosampler, and a Jasco refraction-index detector (Jasco, Tokyo, Japan). A 20 µL volume of the samples was injected and separated on a MetaCarb 67H (300 × 7.8 mm) column at 60 °C, using 0.005 mol L^{-1} H_2SO_4 as mobile

phase at a flow rate of 0.5 mL min^{-1}. Fucose, glucose galactose and rhamose were used as standards.

7.2.6.2 Moisture content on solid substrate

The moisture content of a freshly harvested sample (0.5 g) of fermented solids was determined in accordance with the AOAC method. The sample was dried at 103 ± 2 °C for 2 h, cooled in a desiccator and reweighed.

7.2.6.3 Total sugars and protein content

The total sugars content in the fermented broth was estimated by the anthrone method (Ludwig and Goldberg, 1954) using glucose as standard. This value was used to determine the percentage of substrate consumption. Total protein concentration was determined by the Bradford method (Bradford reagent, Pierce). All absorbance measurements were performed in triplicate.

7.2.6.4 Fucoidanase activity

Fucoidanase activity was determined by measuring the amount of reducing sugars (Miller, 1959) produced from fucoidan through the following reaction: 900 µL of substrate (1% fucoidan from *L. japonica* dissolved with 200 mM acetate buffer, pH 4.5) was mixed with 100 µL of enzyme extract, and the mixture was maintained at 37 °C for 24 h. One unity (U) of fucoidanase activity was defined as the amount of enzyme able to release 1 µmol of reducing sugars per hour under the assay conditions. Inactivated enzyme solution was used as blank.

7.2.6.5 Fucosidase activity

Fucosidase activity was estimated through the following reaction: a substrate solution containing 50 µL of ρ-nitrophenyl-α-L-fucoside 5 mM dissolved with 90 µL of 200 mM acetate buffer, pH 4.5 was mixed with 10 µL of enzyme extract, and the reaction was

maintained at 37 °C for 1 h. One unit (U) of fucosidase activity was defined as the amount of enzyme able to release 1 μmol of ρ-nitrophenyl per hour under the assay conditions.

7.3 RESULTS & DISCUSSION

7.3.1 COMPOSITIONAL ANALYSIS

Chemical composition of untreated, microwave processed, autohydrolyzed, and non isothermal autohydrolyzed algae are presented in Table 7.1. The initial composition of *Fucus vesiculosus* alga (untreated) showed a total sugar concentration of 36.65% (w/w), and low concentrations of protein and phenols (0.110 and 0.59% w/w, respectively). The results obtained for untreated alga were lower than those reported by Rioux *et al.* (2007), the variation can be associated to different factors like the hydrolysis method used for compositional analysis, or the season when the algae were collected.

Characterization of the biomass after hydrothermal pre-treatments revealed higher total sugar content in the autohydrolyzed alga when compared to the microwave processed alga. The highest monosaccharide molar fraction of the studied standards was obtained for fucose with values between 46-62%. The glucose molar fraction showed a significant increment in the pretreated biomasses, correspondent to 1.77, 2.22 and 2.65-fold for MA, HA and NHA, respectively, compared with untreated algae. Chemical profiling demonstrated that the use of pretreated substrates are good prospects for SSF in order to produce enzymes able to hydrolyze fucoidan considering that after the extraction of sulfated fucans by the different hydrothermal processes (results showed in Chapter 4 and 5) the total sugars remained in MA, HA, NHA were 38, 50 and 47% (w/w), respectively, with a high content of fucose.

Table 7.1. Chemical composition of algae substrates for solid state fermentation

				Molar fraction (%mol)			
Algae Substrate	Protein*	Total sugar*	Total phenols*	Glucose	Galactose	Rhamnose	Fucose
UA	0.110 ± 0.01	36.65 ± 0.15	0.59 ± 0.13	6.40	21.26	9.91	62.42
MA	0.093 ± 0.00	14.52 ± 2.74	0.43 ± 0.03	11.37	29.05	7.81	51.75
HA	0.099 ± 0.00	18.71 ± 0.64	0.39 ± 0.11	14.30	30.48	6.73	48.47
NHA	0.092± 0.02	17.02 ± 0.98	0.42 ± 0.07	16.91	29.28	6.88	46.93

UA: Untreated alga; MA: Microwave alga; HA: Autohydrolyzed alga; NHA; Non isothermal autohydrolysis alga

*Yield percentage = mg of protein, total sugar or total phenols / 100 mg alga

7.3.2 EFFECT OF ALGAE SUBSTRATES ON FUNGAL GROWTH

The invasive capacity of two fungal strains was evaluated on three algae substrates in order to indicate the susceptibility to degrade the different solid matrix by these microorganisms. Moreover, the determination of the radial growth rate, as a preliminary assay, allowed establishing the microorganism with higher adaption on the specific media selected for fucoidanase production. Visually, all the evaluated strains showed significant mycelia growth on the different algae substrates, and as can be seen in Table 7.2, highest radial growth was observed with *Mucor* 3P against the values obtained for *A. niger* PSH (around 1.5 fold increase). When compared the growth observed in the solid substrate, the lowest fungal invasion was observed with the untreated alga; also alga-AH substrate showed higher radial rates (0.46 mm h^{-1} *M*.3P and 0.37 mm h^{-1} *A*.PSH) than the growth rates observed on alga-M (0.40 mm h^{-1} *M*.3P and 0.34 mm h^{-1} *A*.PSH).

Table 7.2. Growth kinetic parameters of fungal strains cultivated on algae substrates

Substrate	Strain	U_r (mm/h)	t_d (h)
UA	*Aspergillus niger* PSH	0.11 ± 0.02	8.66 ±0.03
	Mucor sp. 3P	0.20 ± 0.00	8.40 ± 0.36
MA	*Aspergillus niger* PSH	0.34 ± 0.01	9.69 ± 1.91
	Mucor sp. 3P	0.40 ± 0.03	15.72 ± 2.71
HA	*Aspergillus niger* PSH	0.37 ± 0.01	10.53 ± 1.08
	Mucor sp. 3P	0.46 ± 0.02	12.36 ± 1.62

UA: Untreated alga; MA: Microwave alga; HA: Autohydrolyzed alga;

U_r: radial growth rate; t_d: duplicity time

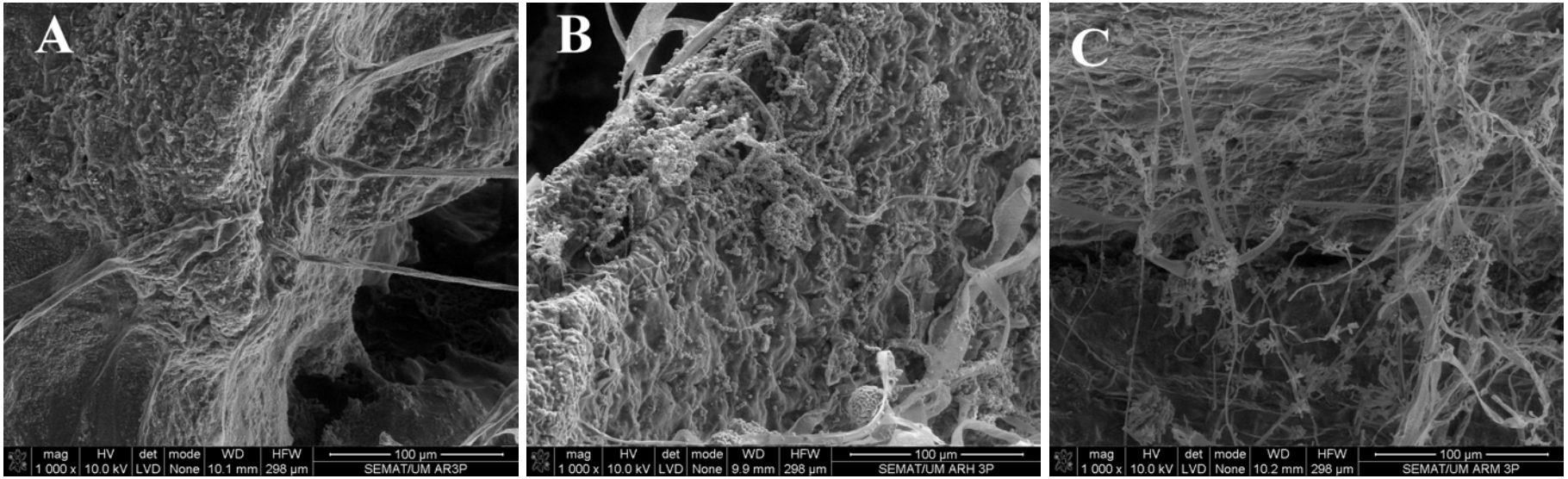


Figure 7.2. Scanning electron micrographs of *Mucor* 3P fungal invasion on algae substrates after 48 h: (A) alga untreated; (B) alga autohydrolysis C) Alga microwave. Magnification: 1000-fold.

The scanning electron micrographs showed in Figure 7.2 allowed explaining radial growth are related with the surface differences, as high roughness and porosity, in algae microwave and alga autohydrolysis, promoting a better micro-environment for the microorganism invasion and penetration. In Figure 7.2A is able to see the low quantity of mycelium present in the algae untreated surface, compared with the huge amount of hyphens and spores observed on alga- microwave and alga-autohydrolysis (Figure 7.2B-C).

It is important to remark that the fungal strains provide by the DIA/UAdeC collection have been widely used in biotransformations of antimicrobial molecules in diverse of vegetal material as pomegranate husk and coffee silver skin with growth rates of 0.41 and 0.20 mm h^{-1}, with *A. niger* PSH (Robledo *et al.*, 2008; Machado *et al.*, 2012).

7.3.3 SOLID STATE FERMENTATION IN ROTATING DRUM BIOREACTOR

Solid state fermentation has been widely studied has a potential process to produce inexpensive enzymes. System as rotating drum bioreactors allowed a continuous mixture to increase the content between the reactor wall and the solid medium and also provides a higher oxygen transfer to the microorganism (Durand, 2003).

The experimental factors to be combined for fucoidan degrading enzyme production were based in the results obtained in previous chapters. Therefore, in Chapter 3 *Aspergillus niger* PSH and *Mucor sp.* 3P were, among the screened strains, the fungus with highest titles of enzymatic activity; and the algae substrates reported in Chapter 4 and 5 were chosen for the high quantity of fucoidan, as a natural inducer source for fucoidanase production. Figure 7.3 showed the cultivation of the filamentous fungus in the horizontal syringe on the rotating drum bioreactor. The mixing was provided by the tumbling motion of the solid medium; and after 5 days of fermentation was visually able to verify the formation of mycelia covering the majority of the substrate surface.

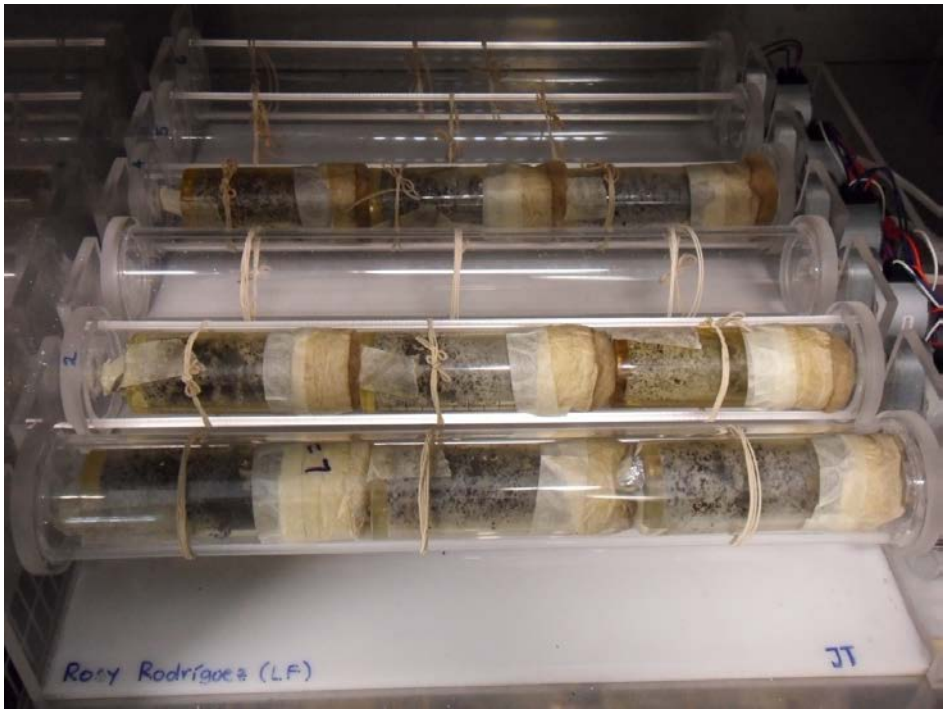


Figure 7.3. Photography of the rotating drum bioreactor with *Mucor* 3P growth on alga-microwave after 96 h.

7.3.3.1 Effect on moisture content

The mixture in SSF bioreactors is associated with the aeration enhancement to create an adequate ambient for microbial growth, however this phenomenon provoked a decrease of substrate free water content. Therefore, the quantitative description of the influence of the moisture changes in solid state fermentation systems is essential for the control of the microbial growth and metabolite production.

Figure 7.4 shows the moisture percentage at different algae substrates after 96 h of fungal fermentation. As can be observed in most of the cases with lack of rotation (0 rpm) the variation of the substrates moisture content is neglible (±3%); only alga-M with *Mucor* 3P culture presented a moisture drop of 15%. On the contrary, the assays performed under rotational conditions (10 rpm) showed a decrease in the moisture content. Algae-AH experiments, with both fungal strains, showed the lowest moisture content (50.49 ± 4.09 and 52.99 ± 0.86 % for *M.* 3P and *A.* PSH, respectively). When evaluating the fungal strains, the highest drops of moisture on algae substrate were

observed with *A. niger* PSH. The moisture lost is also caused by the metabolic heat arising from microbial respiration, as a result of the microorganism growth (Hamidi-Esfahani *et al.*, 2004).

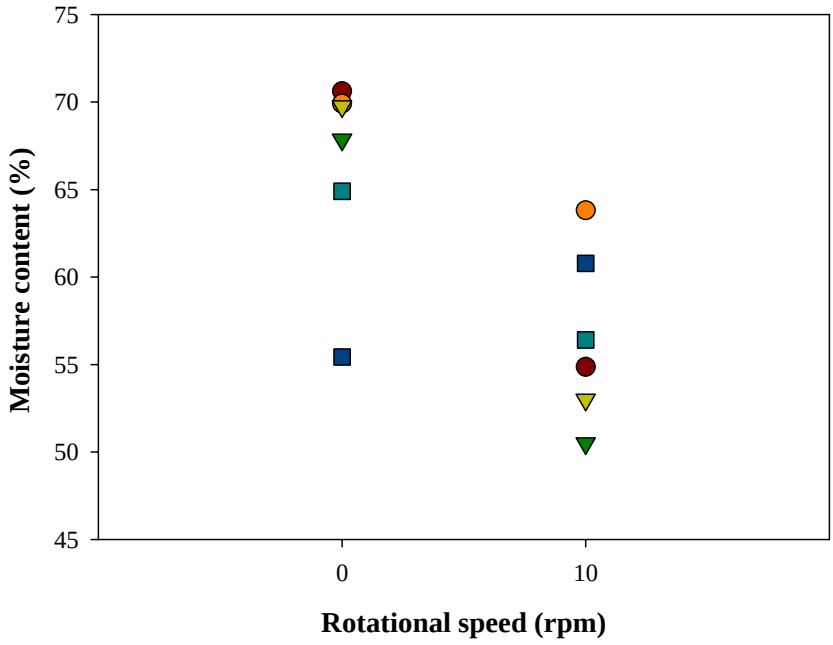


Figure 7.4. Moisture content percentage after 96 h during SSF by *A. niger* PSH and *Mucor* 3P on algae substrates. *A. niger* PSH: ● Alga untreated; ▼Alga autohydrolysis; ■ Alga microwave. *Mucor sp.* 3P: ● Alga untreated; ▼Alga autohydrolysis; ■ Alga microwave.

7.3.3.2 Effect on substrate hydrolysis and sugars consumption

The quantity of total sugars solubilized in the fermentative broth from the different alga substrates in the rotary drum bioreactor was quantified and reported as the fraction of sugars consumption or accumulation from the initial time trough the cultivation period. Figure 7.5A-B shows the profiles obtained under stationary conditions; where the cultures with *Aspergillus* PSH (Figure 7.5A) presented in all the algae substrates a little decrease in the total sugars content before the initial 24 h of fermentation followed by a continuous accumulation until the end of fermentation; for *Mucor* 3P (Figure 7.5B) this behaviour was observed only for untreated algae; alga substrates HA and MA showed a hydrolyzed sugars consumption of 48 and 55%, respectively, reached within 48-60 h.

The effect of the rotational agitation on hydrolyzed sugars intake was more efficient in *Mucor* sp cultures than in *A. niger* PSH cultures (Figure 7.5C-D). For *Mucor* 3P (Figure 7.5D) a constant decrease was observed in the profile for MA sustrate, achieving an sugar intake percentage of 80%; while for HA sustrate the sugars consumption was faster during the first 36 h (54%), after that a possible lack of assimilation, of the hydrolyzed products, by the microorganism caused the increase of sugars concentration.

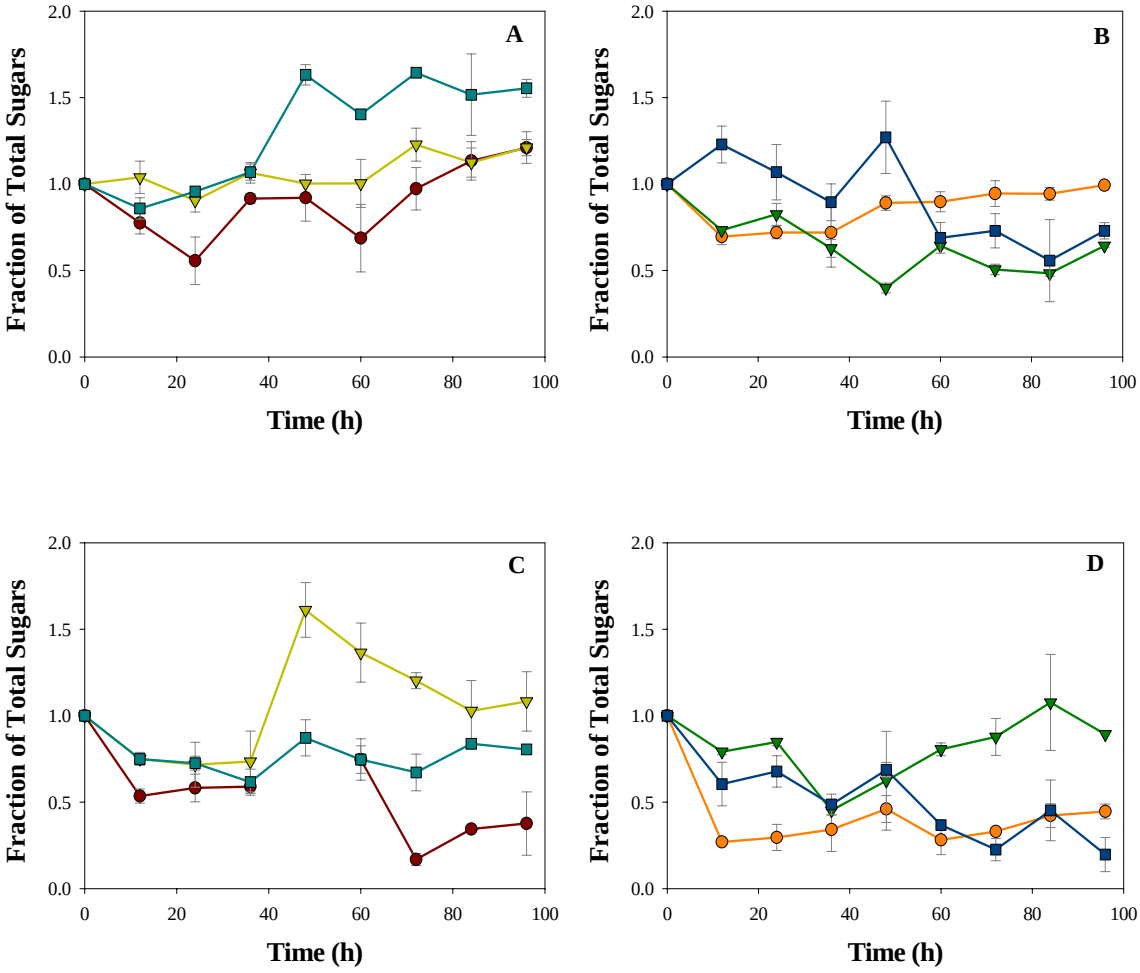


Figure 7.5. Profiles of the solubilized total sugars in the fermentative broth recovery from algae substrates: static (A-B) and rotational (C-D)
A. niger PSH: ● Alga untreated; ▼ Alga autohydrolysis; ■ Alga microwave
Mucor sp. 3P: ● Alga untreated; ▼ Alga autohydrolysis; ■ Alga microwave.

The differences in these results, probably can be explained by the wide range of simple monomer sugars as fucose, rhamnose and galactose provided of different polysaccharides from seaweed. Fucoidan, laminaran, alginate and agar are the mains carbohydrates present in the cell walls of brown alga that acts as source of induction. Hence the only presence of an individual carbohydrates is enough to stimulate the substrate degradation for fungal growth and enzyme production (Giordano *et al.*, 2006). In addition, it is important to consider the wide potential of filamentous fungi to adapt to complex and recalcitrant substrate, as was showed with the use of brown alga; as well as the effect of the fungal morphology closely linked to the properties of the obtained fermentative broth (Grimm *et al.*, 2004)

7.3.3.3 Production of Fucoidan-Degrading Enzymes

SSF experiments using filamentous fungi were carried out with the purpose of producing enzymes with fucoidanolytic activity in the presence of natural inductors present in brown algae (fucoidan, laminaran, alginate and agar). The fermentation time was 96 h and the activity was quantitatively detected by monitoring the release of fucoidan oligosaccharides by conventional reducing assay. Fucoidan hydrolytic enzymes were only analyzed as extracellular metabolites in the culture supernatant, since some studies have reported insignificant presence of intracellular fucoidanolytic activity (Descamps *et al.*, 2006; Rodriguez-Jasso *et al.*, 2010)

In the present SSF experiments, fucoidan hydrolyzing enzymes were not found under static fermentation conditions, only for AM susbtrate; also the fermentative extracts obtained with untreated algae showed non specificity over the fucoidan. From the tested strains, *Mucor* sp. 3P showed better ability to secrete enzymes able to degrade fucan polysaccharide structures..

The fungal cultures that expressed enzymatic activity through the fermentation time are shown in Figure 7.6. As can be seen, the maximum titles of fucoidanolytic activity were achieved after 72 h of cultivation. The best value (3.82 U L^{-1} ; 0.37 U mg^{-1} protien)was obtained with *Mucor sp*. 3P grown on HA sustrate during 72 h. The production profile obtained for this strain showed an increased induction of the enzyme after 24 h;

however after achieved the maximum yield (72 h) the production was decreased, which could be related to the sugars accumulation to the culture media, as discussed before. According to (Descamps *et al.*, (2006) since extracellular enzymes can degrade sulfated fucoidans from algae, it is thus likely that the microorganism attacks the fucoidan structure by cleaving linkages within blocks of alternating α-1→3 and α-1→4 L-sulfated fucose residues.

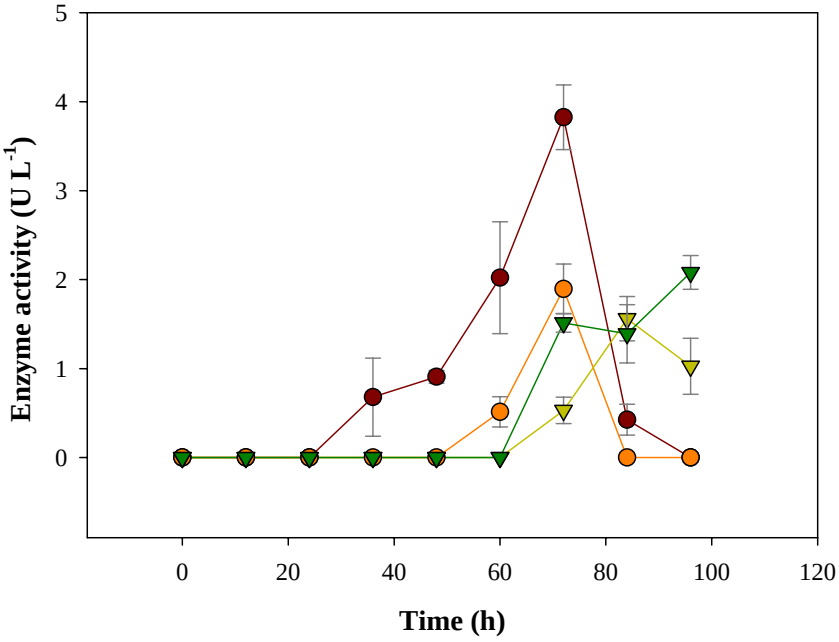


Figure 7.6. Fucoidanase production by *A. niger* PSH and *Mucor sp.* 3P cultivated in algae substrates in a rotating drum bioreactor. *A. niger* PSH: ● Alga microwave (10 rpm); *Mucor sp.* 3P: ● Alga autohydrolysis (10 rpm); ▼ Alga microwave (10 rpm); ▼ Alga microwave (10 rpm).

The other cultures that showed enzymatic activity (1.56-2.08 U L^{-1}) were grown on microwave processed algae. The activity lost after reaching the maximum value was also observed in these experiments, the only exception being for the MA substrate that after 96 h seem to remain the enzyme production.

The results obtained in the current study can be well compared to other reported in the literature. For example, Ivanova *et al.* (2002) and Alexeeva *et al.* (2002) reported titles of 0.4 U L^{-1} and 0.49 U mg^{-1} protein, respectively, with *Pseudoalteromonas sp.* using only fucoidan as substrate. Burtseva *et al.* (2000) isolated enzymes with fucoidan hydrolase activity from 33 species of marine invertebrates, and obtained activities between 0.20 and 0.26 U mg^{-1} protein (Burtseva *et al.*, 2003).

7.3.4 SCALE UP OF THE ROTATING DRUM BIOREACTOR FOR FUCOIDANASE PRODUCTION

The conditions that gave the maximum enzyme activity in the laboratory-scale rotating drum bioreactor (*Mucor* sp. as fungal strain, and algae-AH as substrate) were used in the scale up study, which employed cylinders of 500 mL total volume (Figure 7.7) instead of the 50-mL syringes. In this case, the autohydrolysis pre-treatment of *Fucus vesiculosus* alga, which was previously carried out in an isothermal profile at 180 °C for 20 min; was modified to non isothermal profile at 160 °C, in order to maintain the same severity effect over algae fibers. For comparison, assays using the untreated alga, as well as the AH pretreated alga mixed with an inert support material (synthetic fibers) were also performed.

Establishing the best operational conditions is of great importance to maximize the fucoidan hydrolytic yield. Consequently, the moisture content of the substrates used in the SSF scale up assays was maintained constant throughout the fermentation time by using water or the autohydrolysis liquor. However, no fucoidanase activity was obtained when using AH liquor, which could be related to enzyme-substrate inhibition by other sugars present in the liquor after AH extraction. It is also worth mentioning that non inhibitory compounds (hidroxylmethylfurfural and furfural) were detected in the AH liquor (data not shown). Due to the above mentioned reason, the moisture content of the substrates used in the SSF scale up experiments was adjusted with water, and then, fucoidanase activity was observed (Figure 7.8A).

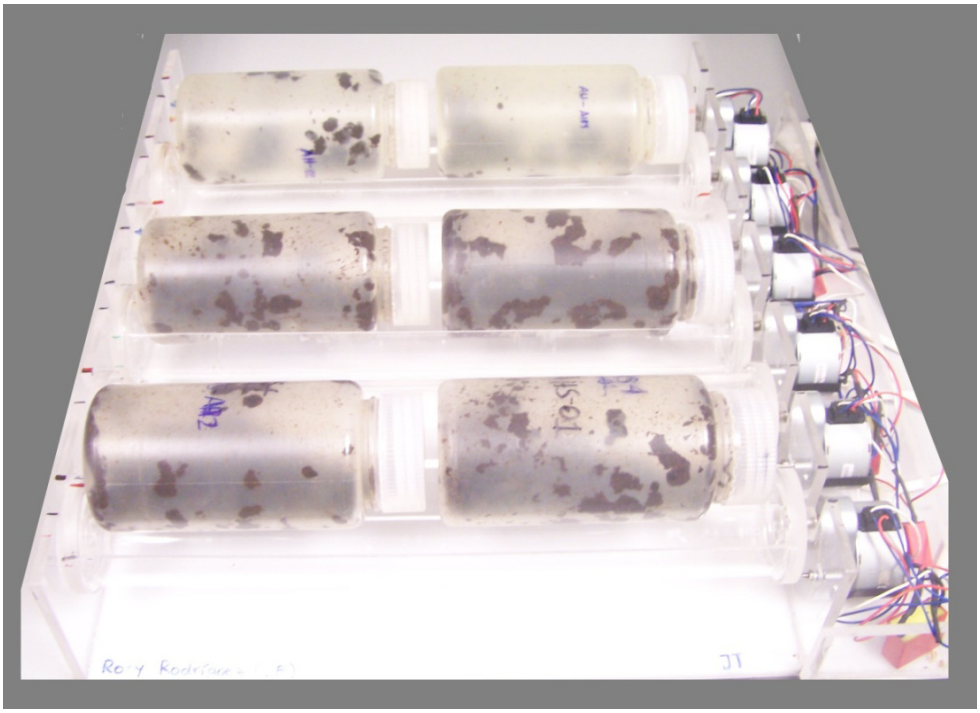
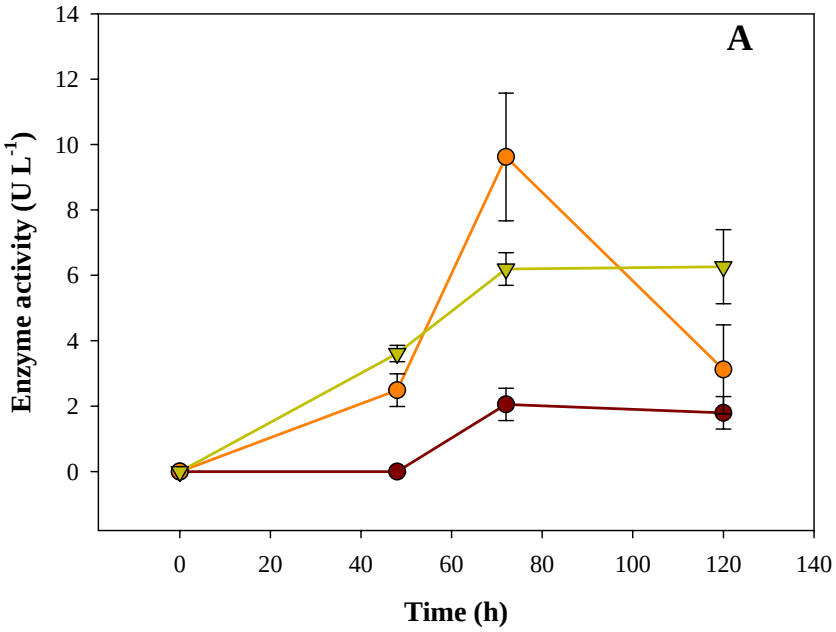


Figure 7.7. Photography of the rotating drum bioreactor with 500 mL cylinder.

The secretion of fungal enzyme showed similar performance along the fermentation time, reaching the highest titles after 72 h. The maximum activity (9.62 ± 1.95 U L^{-1}) was obtained when using NHA substrate; but similar to the small scale experiments, the enzyme production fall down after this time (until six units in the subsequent 48 h), which could be associated to the increase in the concentration of sugars hydrolyzed to the media (Figure 7.8B).

Although the maximum title obtained in the experiment with the combination of algae substrate and synthetic fiber (SHA) was only 6.19 U L^{-1} (3 units lower than with ANH), a stable production was observed in this case until 120 h of fermentation. Additionally, no accumulation peak was observed in the total sugars intake. In fact, inert supports have been reported to improve some of the disadvantages of SSF substrates, such as excessive thickness, low porosity and inefficient nutrient uptake (Marin-Cervantes *et al.*, 2008).



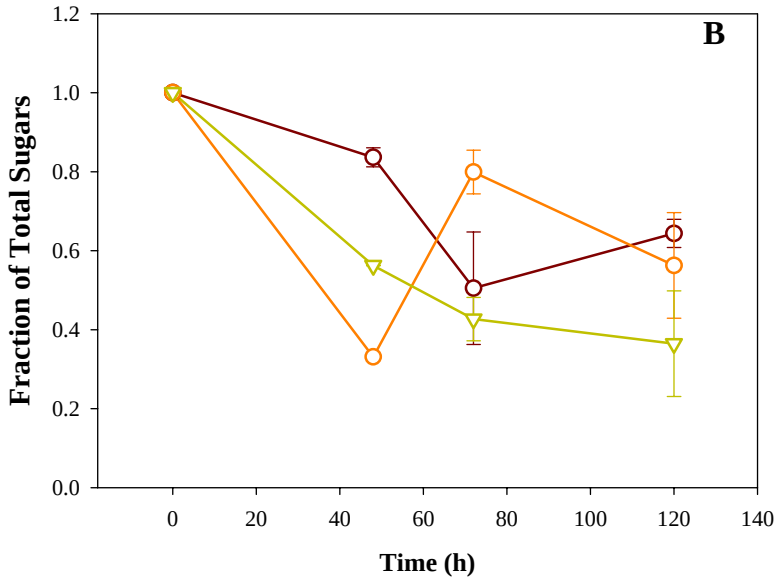


Figure 7.8. Profiles of *Mucor sp* fucoidanase activity (A) and solubilized total sugars (B) in scale up experiment: ●○ untreated alga; ●○ non-isothermal autohydrolyzed alga; ▼▽ non-isothermal autohydrolyzed alga on synthetic fiber cubes.

Considering the algae substrate after autohydrolysis pre-treatment as a rich source of fucose, the evaluation of the induction of α-L-fucosidase by the above experimental condition was evaluated. All the cultures of *Mucor* 3P showed to specificity over the artificial substrate p-nitrophenyl α-L-fucoside (Table 7.3). Among these, the algae substrate supported on synthetic fiber provided the best results; and on the contrary to the factors affecting fucoidanase production, the addition of AH liquor instead of water to the substrate media enhanced the enzymatic titles (from 6.37 to 10.75 U mL^{-1}) and the productivity. Yamamoto *et al.* (1986) investigated the effects of different carbon sources on α-L-fucoside production by *Fusarium oxysporum* and, among several tested monosaccharides, only L-fucose and D-arabinose caused the production of α-L-fucoside, with titles of 8 and 1.5 U mL^{-1}, respectively

Table 7.3. Effect of fucosidase activity induction by SSF on algae substrates

Substrate	Culture media	Maximum activity (U mL^{-1})	Fermentation time (h)
UfA	Water	6.78 ± 1.21	120
	AH-liquor	1.16 ± 0.23	72
NHA	Water	2.94 ± 0.59	120
	AH-liquor	3.33 ± 0.31	120
SHA	Water	6.37 ± 0.11	120
	AH-liquor	10.75 ± 1.06	48

UfA: fresh untreated algae;

NHA: non-isothermal autohydrolyzed alga;

SHA: non-isothermal autohydrolysis alga supported on synthetic fiber

U mL^{-1} = (μmol of p-nitrophenol/h) mL^{-1}

7.4 CONCLUSIONS

Fucoidanase are induced enzymes, produced by media containing fucose and fucoidan. In the present study, algae substrates were used as natural sources for producing fucohydrolytic activity. Moreover, solid state fermentation was selected for fungal bioconversion of brown algae. The designed rotational drum bioreactor showed the critical influence of the substrate homogenization over enzyme production. The set of SSF experiments showed that the fucoidanase production was mainly affected by the mixture conditions of the fermentation process, and *Mucor* sp. strain showed ability to growth and degrade both alga substrates after microwave and autohydrolysis extraction.

The scale up experiments revealed the importance of controlling the moisture content in the solid substrates, and the addition of an inert support such as the synthetic fiber cubes to the alga biomass allowed improving the homogenization of the substrate, increasing the titles of enzyme production. To the best of our knowledge, this is the first study on the application of alga residues as substrate in SSF to produce metabolites of biological interest.

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CHAPTER 8

GENERAL CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

8.1 GENERAL CONCLUSIONS

The main objective of this thesis was the development of a fermentation process for fungal fucoidanases production. In order to cover successfully the thesis aims, several subjects were studied and strategies were implemented. In particular, this work involved the selection of fungal strains and culture conditions on fucoidan media; the extraction and characterization of *Fucus vesiculosus* fucoidan by hydrothermal processes and the induction of fucoidanases production by solid state fermentation. The main contributions of this thesis were the following:

- The identification and morphometric evaluation of three Mexican fungal strains (*Aspergillus niger* PSH, *Penicillium purpurogenum* GH2 and *Mucor sp.* 3P) as microorganisms able to grow over fucoidan media. These strains are the first reports of terrestrial microorganism used to degrade sulphated fucans;

- Liquid fermentation confirms the suitability of *A. niger* PSH to synthesise sulfated fucan-degrading enzymes, reporting the best results with the culture media fucoidan-sucrose;

- Microwave assisted extraction showed to be an effective method to recover sulphated-fucans from *Fucus vesiculosus* maximizing the extraction results to 18% of polysaccharide composed principally of fucose, with the increase of pressure and the decrease of time in the tested conditions (121 psi, 1 min);

- The experimental factorial design and response analysis of fucoidan extracted by the autohydrolysis process allowed the determination of the optimal effect of time and temperature (180°C and 20 min) for the recovery of the highest polysaccharide yield (16.5%) with high contents of fucose and sulphate;

- Both hydrothermal processes evaluated for fucoidan extraction showed to be more environmentally friendly techniques than the most traditional and published procedures, since it do not require the use of chemical solvents and generates less waste, in addition to the reduction in energy consumption;

- Moreover, high antioxidant activities were exhibited with the microwave and hydrothermal sulfated polysaccharides, especially for the $ABTS^{\bullet+}$ assay; this behavior is possibly associated with the high content of sulphate groups;

- With the combination of the selected fungal strains and the *Fucus vesiculosus* algae obtained after microwave and autohydrolysis extraction the fucoidan hydrolytic enzymes were induced by solid state fermentation. The designed rotating drum bioreactor evidenced the relevance of mixture solid systems to increase metabolites production. The highest activity was obtained with *Mucor sp.* 3P and algae after autohydrolysis pre-treatment;

- In the SSF scale up experiments the effect of controlling the moisture content and the addition of inert support on algae substrates improved the system homogenization showing an increment in the enzyme production.

8.2 GUIDELINES FOR FUTURE WORK

Many questions have raised during the development of the present work suggesting some recommendations to improve future work on this field, some of wich are listed below:

a) To optimize the fucoidanases production for developing an efficient protocol for recovery and subsequent the biochemical, physicochemical and catalytical characterization.
b) An evaluation of the genes encoding fucoidanases in the selected fungal strains, in order to produce genetically modified cultures; and also to elucidate the size and genetic composition of the expressed enzymes.
c) Further purification of fucoidan extracts obtained after hydrothermal treatments in order to elucidate the polysaccharides structure and its relation between antioxidant activity mechanisms.
d) Purification of fungal fucoidanases obtained by solid state fermentation; and determination of the enzymes molecular weights, followed by the evaluation of the broad specificities of the purified enzymes over fucoidans from different sources.

The results gathered in this thesis are very promising and have led to interesting new questions that warrant further research.

Scientific output

The overall work presented in this PhD thesis gave origin to the following publications:

PAPERS IN JOURNALS WITH PEER REVIEW

- **Rodríguez-Jasso R.M.,** Mussatto S.I., Pastrana L., Aguilar C.N. and Teixeira J.A. 2010. Fucoidan-degrading fungal strains: screening, morphometric evaluation, and influence of medium composition. *Appl. Biochem. Biotechnol*.162: 2177–2188.

- **Rodríguez-Jasso R.M.,** Mussatto S.I., Pastrana L., Aguilar C.N. and Teixeira J.A.. (Manuscript accepted with corrections). Microwave-assisted extraction of sulfated polysaccharides (fucoidan) from brown seaweed. *Carbohyd. Polym.* 86: 1137-1144

- **Rodríguez-Jasso R.M**., Ortiz I., Mussatto S.I., Pastrana L., Aguilar C.N. and Teixeira J.A. (2010). Optimization of sulphated polysaccharides recovery from brown seaweeds by microwave-assisted extraction. J. Biotechnol. 150S: S394-395.

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- **Rodríguez-Jasso, R.M.,** Teixeira J.A., Aguilar-Gonzalez, C.N., Pastrana, L. 2008. Identification and evaluation of fungal strains with fucoidan degradation potential. *Proceedings of the 10th International Chemical and Biologial Engineering-CHEMPO*R, Braga, Portugal, 4-6 September, pp: 2106-2109 (in CD-ROM)

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- Rodríguez-Jasso R.M., Ruiz H.A., Mussatto S.I., Pastrana L., Aguilar C.N. and Teixeira J.A. Extraction of sulfated polysaccharides by autohydrolysis of brown seaweed *Fucus vesiculosus*.