Enzyme-assisted extraction (EAE) for the production of antiviral and antioxidant extracts from the green seaweed *Ulva armoricana* (Ulvales, Ulvophyceae)

Hardouin Kevin 1,2, *, Bedoux Gilles 1, Burlot Anne-Sophie 1, Donnay-Moreno Claire 4, Bergé Jean-Pascal 5, Nyvall-Collén Pi 3,5, Bourgougnon Nathalie 1

1 Univ Bretagne Sud, LBCM, IUEM, EA 3884, F-56000 Vannes, France.
2 SA OLMIX, ZA Haut Bois, F-56580 Brehan, France.
3 Amadeite SAS, Pole Biotechnol Haut Bois, F-56580 Brehan, France.
4 IFREMER, Lab BIORAFhe, Rue Ile Yeu,BP 21105, F-44311 Nantes 03, France.
5 IDMer, 2 Rue Bateliere, F-56100 Lorient, France.

* Corresponding author : Kevin Hardouin email address : kevin.hardouin.pro@gmail.com

Abstract :

In order to develop innovative methods for the production of bioactive extracts with potential health benefits, enzyme-assisted extraction has been applied for the bioconversion of *Ulva armoricana* (Ulvales, Ulvophyceae). The extraction yields, chemical composition, the antioxidant and antiviral activities were determined to assess the efficiency of six commercial enzymatic preparations. Endo-protease treatments significantly increased the extraction yields compared to the control. The organic matter, neutral sugar and protein contents were increased in all extracts compared to an extraction with water, up to 2.0-fold, 2.7-fold and 1.75-fold, respectively. The samples prepared by a multiple-mix of glycosyl-hydrolases and an exo-β-1,3(4)-glucanase revealed activities against *herpes simplex virus* type-1 at the median effective concentration (EC₅₀) of 373.0 ± 20.7 and 320.9 ± 33.6 μg/ml, respectively. These activities were correlated to high amounts of rhamnose, uronic acids and sulfate groups which are the main constituents of ulvans. Free radical scavenging capacity at medium inhibition concentrations (IC₅₀) of 1.8 and 12.5 mg/ml was shown for the extracts produced with endo-protease treatments and 6.0 mg/ml for the sample resulting from the extraction with the multiple-mix of glycosyl-hydrolases. This study reports the antiviral and antioxidant activities of *Ulva armoricana* extracts produced by enzyme-assisted extraction.

Keywords : Ulvan, Antiviral activity, Carbohydrate, Endo-protease, Bioconversion, Biorefinery
1. Introduction

Macroalgal blooms occur worldwide, mainly caused by species from the phyla Rhodophyta [1] and Chlorophyta [2], but the most widespread are caused by the Chlorophyta species, such as those within the genus *Ulva* Linnaeus [3]. During the last few decades, the coasts of Brittany (France) have suffered from significant green tides mainly constituted of non-attached populations of *Ulva armoricana* and *Ulva rotundata* (Ulvophyceae) [2]. Many studies have addressed the ecological and environmental consequences of green tides which include uncoupled biogeochemical cycles, decreased water transparency, degradation of the intertidal environment, reduced biodiversity, hypoxia or anoxia, destruction of the marine coastal habitat (e.g., seagrass beds) and economic losses to marine industries [3]. However, these seaweed beachings also represent a huge algal biomass which could be exploited, for applications as plant elicitors, in animal health and in feed [4,5,6].

At this time, harvesting of these seaweeds remains the most cost-effective way to obtain a high tonnage of biomass. However, as the resource is unpredictable, aquaculture can be an alternative in order to ensure its availability and improve the quality and homogeneity of the resource.

Over the last few years, there has been more investigations in the research into new bioactive natural molecules and valuable metabolites from seaweeds having an economic impact [4,7]. Several biological activities such as antiviral [6], antibacterial [8], antioxidant [9], anticoagulant [10] and antitumor [11] have been identified in red (Rhodophyta) and brown (Phaeophyta) algae but few studies have been reported on
green algae (Chlorophyta). Therefore, the production of bioactive extracts could represent a potential application for the valorization of algal blooms. The algal cell wall is composed of two main classes of polysaccharides: (1) cellulose, hemicelluloses, mannans and xylans which constitute the skeleton and (2) a species-specific matrix phase of water soluble polysaccharides [12, 13]. In Ulva sp., the matrix phase contains ulvans composed of sulfated (sulfation 17 %) xyloarabinogalactans and sulfated (sulfation 22 %) glucuronoxylorhamnans containing 20 % of uronic acids [14,15]. Lahaye and Robic [13] proposed that the two major repeating disaccharides of the glucuronoxylorhamnans are aldobiuronic acids designated as type A: ulvanobiuronic acid 3-sulfate (A3s) and type B: ulvanobiuronic acid 3-sulfate (B3s). Partially sulfated xylose residues at O-2 can also occur in place of uronic acids. In addition, glucuronic acid can branch at O-2 of rhamnose 3-sulfate [15]. Traditionally, hot water, acids, bases, organic solvents or ultrasounds were used to extract molecules from most bioresources; however, the enzymatic hydrolysis, an effective and nontoxic procedure, is currently applied in various industrial applications. Manufactured proteases are used in animal feed to improve the digestibility of proteins and in the detergent industries. Glycosyl hydrolases are applied in food/vegetable and starch processing, brewing, paper and pulp industries. For the biorefinery of macroalgae, there is a trend to isolate new specific enzymes from marine organisms, for the degradation of polysaccharides, like ulvan-lyases, glucuronan-lyases, laminarinases, agarases, carrageenases or porphyranases [16]. These enzymes have been isolated and produced at laboratory scales but they are not yet available for industrial purposes. In this study, in order to develop an industrially applicable technology, commercial enzymatic preparations have been selected.
Enzyme-based processes, using non-selective enzymes, have already been applied for
the production of bioactive extracts from macroalgae. Anticoagulant activities,
antioxidant capacity and immunomodulation properties have been highlighted in
enzymatic extracts of green, brown and red seaweeds, respectively [17].

The aim of this work was to evaluate six non-selective commercial enzymatic
preparations, two proteases and four carbohydrases as tools for improving the extraction
efficiency and producing bioactive fractions from the French green seaweed \textit{U. armoricana}. The extracts were characterized by their respective biochemical
composition. Antiviral and antiradical activities were studied on both crude extracts and
their isolated polysaccharide fractions.

2. Material and Methods

2.1 Materials

\textit{Algal sample: Ulva armoricana} (Ulves, Ulvophyceae) was collected on the beach in
Plestin-les-Grèves (48°39'28'' N, 3°37'47'' W), English Channel (Brittany, France), on
June 18\textsuperscript{th}, 2012. Algae were then washed with tap water, ground and frozen at -25°C.

\textit{Enzymes:} Six commercial enzymatic preparations, provided by Novozymes (Bagsværd,
Denmark), were used for the hydrolysis of seaweed, a neutral endo-protease (P1, EC
3.4.24.28), a mix of neutral and alkaline endo-proteases (P2, EC 3.4.24.28/EC
3.4.21.62), a multiple-mix of carbohydrases (C1, EC 3.2.1.6/EC 3.2.1.8/EC 3.2.1.4/ EC
3.1.1.73), a mix of endo-1,4-β-xylanase/endo-1,3(4)-β-glucanase (C2, EC 3.2.1.8/EC
3.2.1.6), a cellulase (C3, EC 3.2.1.4) and an exo-β-1,3(4)-glucanase (C4, 3.2.1.58). The
EAE were compared to a control (Blank), treated in the same conditions and without enzyme. The blank corresponds to a classical water extraction at 50 °C for 3 h.

2.2 Enzyme-Assisted Extraction

Extractions were performed in a bioreactor in which 500.0 g of crushed algae (8.3 % dry matter, \( dm \)) and 500.0 g of Milli-Q water were mixed. The enzyme preparations (6 % weight/dry weight, \( w/dw \)) were added to the algae and allowed to react during 3 h at 50°C followed by denaturation at 90°C for 15 min. The pH of the reaction ranged from 6.2 at the beginning of the reaction to 5.9 at the end. After extraction, the samples were filtered, on a Buchner system using a synthetic cloth, and then centrifuged (8,000 g) for 20 min at 20°C. In the end, two fractions were obtained, a soluble extract and an insoluble pellet. All samples were then freeze-dried and stored at 4°C. Every extract consisted of the addition of the filtered soluble fraction plus the residual soluble fraction retained in the pellet.

The addition of dry matter, proteins, carbohydrates etc. due to the addition of the enzymatic preparations have been taken into account in the calculation both of extraction yields and biochemical composition of the hydrolyzates.

2.3 Biochemical analyses

An aliquot of dried seaweed (10.0 mg) was hydrolysed for 2 h at 100°C with 1 M hydrochloric acid (1.0 ml) for the characterization of the raw material and then neutralized with 1 M sodium hydroxide (1.0 ml). The methods used for the biochemical
analyses were the phenol sulfuric acid method of Dubois for neutral sugars, the meta-hydroxy-di-phenyl (MHDP) method for uronic acids, the Azure A method for sulfate groups and the bicinchoninic acid (BCA) protein assay kit for total proteins. All these methods are described and detailed in Hardouin et al. [18]. The total phenolic content was quantified by spectrophotometry according to the Folin Ciocalteu method [19] with minor modifications. One milliliter aliquot of extract solution (concentration range from 1 to 5 mg/ml) was mixed with 5 ml of Folin Ciocalteu reagent (10 % in distilled water). After 5 min, 4 ml of sodium carbonate (7.5 % in distilled water) were added. The samples were incubated for 2 h at room temperature in darkness. The absorbance was measured at 760 nm. A standard curve with serial gallic acid solutions (0 to 500 µg/ml) was used for calibration. Results were expressed as g gallic acid equivalents (GAE) per kg of extract.

2.4 Amino acids composition

The total amino acid composition of freeze-dried extracts was determined after hydrolysis in 6 M HCl at 118°C for 18 h. The hydrolyzed samples were completely dried under a nitrogen atmosphere and diluted by adding 2.5 ml of water. The amino acid samples were prepared according to the EZ faast™ (Phenomenex, Torrance, California, U.S.A) procedure consisting of a solid phase extraction step followed by derivatization and liquid/liquid extraction. The amino acid samples were analyzed on an autosystem Gas chromatography–Flame ionization detection (Perkin Elmer, France; GC-FID) system composed of a Zebron ZB-AAA GC column (10 m x 0.25 mm, Agilent, CA, USA). 2 µl of samples were introduced in the injector (250°C) and
separated using the following program: 110°C to 320°C (increase of 32°C/min). The
detector was at 320°C and the gas pressure at 1.7 bar. The signals were registered using
the Galaxie software (Varian, CA, USA). The amino acids were quantified by their
response factor relative to the internal standard Norvaline added at a concentration of
200 μmol/l.

2.5 Sugar composition

The composition of unitary sugars present in the polysaccharide chains was determined
by High Pressure Anion Exchange Chromatography (Dionex). Prior to analyses, 1.0 ml
(2 mg/ml) samples were hydrolysed for 48 h at 100°C with 110 µl of 1 M hydrochloric
acid in a sealed glass tube. Before filtration, the samples were neutralized with 1 M
sodium hydroxide. 30 µl were injected on an analytical column (CarboPac PA1, 4 x 250
mm) with elution, at 1 ml/min and 110 bars, performed in alkaline conditions. Solution
A consisted of Milli-Q water; solution B was 0.1 M sodium hydroxide and solution C
was 1 M sodium acetate/0.1 M sodium hydroxide. The elution program corresponded
to: 20 min isocratic conditions (solution A/solution B, 80:20), linear gradient (solution
C 100 %, 0 to 5 min), 5 min isocratic conditions (solution C, 100 %) and 20 min
isocratic conditions (solution A/solution B, 80:20). Sugars were detected by pulsed
amperometry with a detector composed of a silver standard electrode and a gold
working electrode. The chromatograms were treated using Chromeleon® software
provided by Dionex. Carbohydrates were identified and quantified by using calibration
curves of standards composed of ribose, glucose, rhamnose, galactose, arabinose,
xylose, mannose, fructose, guloheptose and glucuronic acid.
2.6 Polysaccharides molecular weight (Mw) distribution

The polysaccharide molecular weight (Mw) distribution was determined by High Performance Steric Exclusion Chromatography (HPSEC). The analytical chain was composed of an auto sampler (ASI 100, Dionex), an analytical pump (P680, Dionex) and two detectors connected in series, a UV detector set at 280 nm (UVD 400, Dionex) and a refractometer (Iota 2, Precision Instrument). Samples were prepared at 1.0 mg/ml in the eluent and filtered at 0.45 µm. The separation system consisted of a TSKgel® GMPW standard Guard Column (TOSOH) and a TSKgel® GMPWXL column (TOSOH 300 x 7.8 mm, 13 µm). Elution was carried out at 30°C, by using 0.1 M sodium nitrate (NaNO₃) at a flow rate of 0.5 ml/min. Dextran standards with molecular weights of 5.3 kDa, 17.9 kDa, 129 kDa and 636.4 kDa, 1 % (w:v), were used for calibration. Sodium chloride (NaCl) was used to determine the maximum retention time (total volume, Vᵣ) of the column.

2.7 Polysaccharide isolation

The isolation of polysaccharides was performed by ethanol precipitation. 450 ml of ethanol were added to 50 ml of extract and stored at 4°C over-night. Precipitates were recovered by centrifugation (8,000g) at 4°C for 20 min, washed twice with cold ethanol and freeze-dried.

2.8 Evaluation of biological activities
In vitro antiviral and cytotoxicity evaluation by cell viability: The *in vitro* antiviral and cytotoxicity evaluation were performed according to the method used by Hardouin et al. [18] on the model *Vero* cells and *Herpes simplex* virus-type 1 (HSV-1).

DPPH free radical scavenging assays: The antiradical activities of extracts were measured with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [20]. A solution of DPPH was prepared at 0.25 mM (0.1 g/l) by diluting 10 mg of DPPH in 100 ml of methanol. Butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT) were used as standards (2 to 50 µg/ml). The samples were dissolved in water at 1, 5, 12.5 and 25 mg/ml and aliquots of 1 ml were mixed with 1 ml of DPPH reagent. The samples were incubated for 30 min at 40°C and the OD measured at 517 nm was compared to a blank of methanol and water. The inhibitory concentration (scavenging) was calculated by the formula: Scavenging (%) = [(OD\textsubscript{control} − OD\textsubscript{sample}) / OD\textsubscript{control}] × 100. The IC\textsubscript{50} was determined with the curve and correspond to 50% inhibition.

2.9 Statistical analysis

Results are expressed as means ± standard deviation (SD) (n = 3). The statistical analysis was carried out on SPSS (IBM, Armonk, NY, USA) using the one-way analysis of variance (ANOVA) followed by a Duncan test at the 5% level (p < 0.05) to evaluate differences between the samples. For each series of values, the significant differences are labelled by superscript letter.

3. Results and discussion
3.1 Characterization of the *Ulva armoricana* raw sample

*Ulva armoricana* samples contained 15.9 ± 1.2 % ash, 23.4 ± 1.4 % neutral sugars, 21.1 ± 0.1 % uronic acids, 24.4 ± 0.1 % proteins, 4.0 ± 0.1 % total nitrogen, 20.9 ± 0.9 % sulfate groups, 0.6 ± 0.1 % of total phenolic compounds and 0.6 ± 0.0 % lipids. The total carbohydrate content (64.4 %) was calculated by the addition of neutral sugars, sulfate groups and uronic acids values. Lipids and phenolic compounds represented a very small part of the algal composition. These levels are in agreement with those reported by Holdt and Kraan [7] for the genus *Ulva* sp. This composition also confirmed that polysaccharides are the main constituent of the *Ulva* sp. thallus. The respective contents of each metabolite in algae vary greatly depending on season, state of growth, geographical area and environment quality [21].

3.2 Enzymatic hydrolysis yields

Figure 1 shows the amount of dry matter in the extracts, expressed as a percentage of the total seaweed dry weight (% total dw). The proportion of the dry matter solubilized by grinding and incubation was estimated by the blank at 44.3 ± 0.5 % of the initial seaweed dry weight. The difference between this value and values obtained after enzymatic treatment correspond to the benefits of the enzyme-assistance. The extraction yields, 44.9 ± 1.7 % for C1, 44.6 ± 0.6 % for C2, 45.1 ± 2.7 % for C3, were not significantly different from the blank and show that enzymes C1 (mix of carbohydrases), C2 (endo-1,4-β-xylanase/endo-1,3(4)-β-glucanase) and C3 (endo-1,4-β-
glucanase) did not improve the extraction. The enzymes C4 (exo-1,3(4)-β-glucanase), P1 (neutral endo-protease) and P2 (neutral and alkaline endo-proteases) induced a significant increase in the amount of solubilized dry matter, with extraction yields of 70.7 ± 1.1 %, 76.7 ± 0.8 % and 88.4 ± 0.2 %, respectively. These three enzymes provided an extraction gain of 60 %, 73 % and 100 %, respectively, compared to the control (blank).

Previous studies have reported the extraction of water-soluble compounds from different edible seaweeds belonging to the genus *Ulva* sp [14,22,23,24]. The yields and the chemical nature of the isolated compounds are greatly dependent on the extraction procedures.

Aqueous and chemical extractions were applied in order to produce fractions enriched in different metabolites. Co-extraction of the water-soluble polysaccharides (ulvans and glucuronans), using oxalate solution at 85°C, led to an 8-29 % extraction yield for *Ulva* sp. [12,13,22]. Xyloglucans and glucuronans, two minor parietal polysaccharides, have also been reported to be selectively isolated in an alkali-soluble phase [13,14]. The application of a basic (0.1 M NaOH) solution containing β-mercaptoethanol produced the highest protein yield for the species *Ulva rigida* and *Ulva rotundata* [22]. In this latter study, the use of enzymatic preparations (cellulase and polysaccharidases mixture) appeared to be less efficient than aqueous solutions procedures. In this work, the lack of effect of the three carbohydrase preparations C1, C2 and C3 agrees with data observed by Fleurence et al. [22]. The enzymatic preparation C4 has been reported to exhibit a proteolytic side-activity [25] that may explain the improved yield, only slightly below those of the proteases. This is the first report of enzymatic degradation of *Ulva* sp. using proteases. The high efficiency of the proteolytic activities can be explained by
the ubiquitous nature of proteins and illustrates the importance of proteins for the
integrity of the cell wall. Finally, the nature of enzymatic activity greatly influenced the extraction yields which
are higher than those obtained by chemical methods [26,27]. According to these results, enzymes can be classified in two categories: enzyme preparations C1, C2, C3, which have low efficiencies and enzyme preparations C4, P1 and P2 which have a higher extraction power. These last three enzymatic preparations are also more efficient than classical water and chemical methods. The selectivity of the enzyme-assisted extraction was assessed by the characterization of the biochemical composition.

3.3 Biochemical characterization of crude enzymatic extracts

Table I shows the biochemical composition of extracts, after deducting the input from the enzymatic preparations. The biochemical composition is expressed as the percentage in the dry hydrolyzates and by the absolute weights (g) solubilized by the process. The ash contents (% \textit{dw}) ranged between 16.7 ± 2.1 and 18.6 ± 2.1 and were consistent with the content found in the raw material. These levels correspond to the inorganic part of the seaweed. They were increased in samples C4, P1 and P2 with a good correlation with the extraction yields. The same trend was observed for total phenolic compounds, indicating that the extraction of non-structural components (ash, total phenols) is correlated with the extraction yields with no apparent effect of the extraction procedure. The neutral sugars contents were higher in samples C1 and C3, 18.4 ± 0.8 and 18.3 ± 3.3 \% \textit{dw}, respectively. Conversely, the blank and the extract C2 contained the lowest amounts, respectively 9.0 ± 1.2 and 8.1 ± 1.6 \% \textit{dw}. Meanwhile, the extracts obtained
from the enzymes C4, P1 and P2 contain intermediate contents between $11.3 \pm 2.3$ and $14.7 \pm 0.5 \%$ \textit{dw}. However, the absolute amount of neutral sugars solubilized by these three enzymes is higher with respectively $3.7 \pm 0.2$, $4.1 \pm 0.2$ and $3.7 \pm 0.8$ g. The neutral sugars contents were lower than those determined after chemical extraction methods from \textit{Ulva clathrata} [27] and after aqueous extraction from \textit{Ulva rotundata} [26].

The blank, C2 and P1 contained the highest protein concentrations with $9.4 \pm 0.4$, $8.8 \pm 0.9$ and $10.1 \pm 0.3 \%$ \textit{dw}$\,$ respectively, but the highest total protein weights were observed in samples C4 ($2.2 \pm 0.1$ g), P1 ($2.8 \pm 0.1$ g) and P2 ($2.1 \pm .01$ g). The increase in total protein weights of these samples is directly correlated with the increase in extraction yields.

From all extracts, samples C1 and C3 containing $53.7 \%$ and $55.6 \%$ \textit{dw} of total carbohydrates (neutral sugars, uronic acids and sulfate groups), respectively, were the most concentrated. On the opposite, the samples C4, P1 and P2 contained the lowest concentrations, $46 \%$, $46 \%$ and $36 \%$ \textit{dw}$\,$ respectively. Enzymatic preparations C1 and C3 appear to be the most selective for the extraction of total carbohydrates. However, due to the significant increase in extraction yields, enzymes C4, P1 and P2 solubilized a larger amount (g) of total sugars and protein, and are the most efficient. The proteolytic side-activity of the enzymatic preparation C4 [25] is confirmed by the selective extraction of proteins.

Due to the mainly polysaccharidic nature of the cell wall of \textit{U. armoricana}, these results may seem paradoxical, but they confirm that non-selective industrial carbohydrates do not hydrolyze the matricial polysaccharides of the seaweed cell wall and therefore do not improve the extraction of seaweed components. Conversely, to explain the efficient
effect of proteases on seaweeds, the first assumption is that proteases have a
destabilizing effect on the cell wall, which causes the release and solubilization of a
higher amount of compounds. These results also suggest that proteins are attached to
polysaccharides and act as a cement in the structure of the cell wall [13]. However,
there is a second hypothesis which is that proteases hydrolyze the membrane cell
proteins causing a release of intracellular material and not exclusively molecules from
the seaweed cell wall.

3.4 Amino acid composition of extracts

Gas chromatography-Flame ionization detection (GC-FID) was used to identify and
quantify 16 amino acids (AA). Cysteine, arginine and tryptophan were not determined
by this method and glutamine and asparagine were quantified as glutamic and aspartic
acids, respectively. The results were expressed as a percentage of the total AA
measured. The average rates of AA released in the extracts are given in Table II. The
characterization of the AA profile revealed minor differences between extracts. AA can
be divided into three categories according to their content in samples, high (< 10 %),
medium (3-10 %) and low (> 3 %).

These results provided information on the protein composition of the algae. Four AAs
were identified as major, alanine, glycine, aspartic acid and glutamic acid as they
constituted more than 10 % of the extracted proteins. Methionine, hydroxyproline,
lysine, histidine and tyrosine were present in very small amounts, ranging from 0.9 to
2.9 %. The content of the remaining AAs was average, between 3 and 10 %. Although
the composition of the samples was quite similar, there were still some differences
against the blank. The alanine content was between 12.1 % and 14.1 % against 16.3 % in the blank. The results were similar for glycine with rates between 8.7 % and 9.1 % in the extracts against 11.1 % in the control. The trend was reversed for glutamic acid and aspartic acid as their rates in the negative control, 8.1 % and 11.1 % respectively, were lower than in the extracts, 9.3 to 14.3 % and 12.9 to 14.5 %, respectively. No difference appeared between extracts and the blank for the other AAs. Therefore, due to the differences in the amounts of several amino acids, the proteins solubilized by the enzymatic treatment seem to have a different composition from those solubilized by incubation in water. The nature of the enzymatic preparation did not affect the amino acid profile of the extracts. These results matched with those obtained by Fleurence et al. [28], who had shown the enriched composition, in alanine (5.51 – 7.01 % proteins), glycine (6.34 – 7.53 % proteins), aspartic acid (6.09 – 11.84 % proteins) and glutamic acid (11.70 – 23.35 % proteins), of proteins from *U. armoricana* collected in October 1997. However, some slight differences appear in the respective amino acids contents. These differences could be due to the seasonal variability and the life cycle of seaweed which do not produce the same types of proteins during the year. These differences could also be explained by the extraction method used. Thus, enzyme-assisted extraction might selectively extract different types of protein. The seasonal variability of the proteins and amino acids composition was also highlighted by Fleurence et al. [28].

### 3.5 Sugar composition
The results from the compositional analyzes of carbohydrates by High Pressure Anion Exchange Chromatography (HPAEC) are given in Table III. Values represent the mean of the percentage of the different sugars relative to the total carbohydrate content (g/100 g total carbohydrate). Fructose and ribose were identified in very low concentration (< 3% total sugars) while some unidentified and minor carbohydrates are included in ‘other sugars’. Two unidentified and charged carbohydrates were added to uronic acids (UAc) due to their retention time, close to the glucuronic acid. These were probably galacturonic and iduronic acids [13].

Analyzes showed the predominance of glucose, rhamnose and uronic acids. Lahaye and Robic [13] described the composition of ulvans and showed that it is composed mainly of rhamnose (16.8 - 45.0 % dw), xylose (2.1 - 12.0 %), glucose (0.5 - 6.4 %), uronic acid (6.5 - 19.0 %), and sulfate groups (16.0 - 23.2 %). The results obtained in our study correspond to these values and show the presence of ulvans in the extracts. Lahaye and Robic [13] also reported the presence of β-glucans, polymers of glucose, which could explain the high levels of glucose in crude samples (Table III a).

The isolation step aimed to selectively separate the polysaccharide fractions from the extracts. The sugar compositions of these fractions (Table III b) differed from those of the crude hydrolyzates. The precipitation led to a decrease of the glucose amounts in all samples, highly amplified (-47 to -72 %) in C1p, C2p, C3p, and C4p. The isolation also corresponded to a high increase in the rhamnose and uronic acids contents in the same samples. These variations were lower in the blank and in samples P1p and P2p.

These results suggest that the proteases and the control led to the solubilization of neutral (β-glucans) and matricial (ulvans) carbohydrates in their native forms, without degradation. Regarding the loss of glucose after precipitation, β-glucans might be
degraded by carbohydrases C1, C2, C3 and C4 (which contain β-glucanases) in oligosaccharides and in monosaccharides which were not precipitated, while the ulvans were recovered in their native forms. Combined with the extraction yields, these results show that carbohydrases have a selective action on the glucans of the *U. armoricana* cell wall, without improving the efficiency of the extraction, whereas proteases, which were more efficient, solubilized native carbohydrates.

The samples obtained with the enzymatic preparation C4, which was one of the most efficient enzyme mixtures, show a decrease in glucose and an increase in rhamnose and uronic acid contents, confirming the simultaneous effect of the β-glucanase and the protease previously observed in this study.

### 3.7 Polysaccharides molecular weight (Mw) distribution

The molecular weight distribution of poly- and oligosaccharides in extracts has been determined by High Performance Steric Exclusion Chromatography (HPSEC). Figure 2 is a representative example of the chromatograms obtained for the different samples, comparing the samples C4 and C4\(_p\), respectively, before and after ethanol precipitation. The dotted line represents the crude extract C4 and the solid line the polysaccharide fraction C4\(_p\). The figure shows four peaks in C4 and three in C4\(_p\). The last peak (C4) was the total volume of the column and corresponded to salts. This peak does not appear on chromatogram C4\(_p\), confirming that salts and monosaccharides were eliminated by the precipitation. The high mass polysaccharide fractions increased in isolated samples while the low mass polysaccharides decreased. These results confirmed the efficiency of ethanol precipitation for the isolation of polysaccharides.
Chromatograms also show that there were two groups of polysaccharides, with average molecular weights of 2,000 kDa and 600 kDa, and a group of oligosaccharides (3 kDa). The two groups of polysaccharides confirmed the presence of ulvans in samples and correspond to the results reported by Robic et al. [29] describing the structure of ulvans. The same study also described the molecular weights of ulvans ranging from 300 to 500 kDa for the first group and from 140 to 180 kDa for the second group. These values are below those obtained in our study. This difference may be due to the extraction method or HPSEC conditions. In their study, Robic et al. [29] used water extraction in sodium oxalate at 85°C for 2 h, which is described as the optimal condition for ulvan extraction. The increase of polysaccharides and the corresponding carbohydrate compositions confirm the enrichment in ulvans in the isolated fractions.

3.8 Screening of in vitro antiviral activity by cell viability of extracts

The crude extracts and the isolated polysaccharide fractions were tested in vitro for their cytotoxicity on Vero cells and their antiviral activity against herpes simplex virus type-1 (Table IV).

Acyclovir, used as reference molecule, presented an effective concentration (EC_{50}) of 0.3 µg/ml and a cytotoxicity (CC_{50}) above 500 µg/ml. No crude extracts exhibited cytotoxic effect to Vero cells for concentrations up to 500.0 µg/ml. The microscopic examination and the assessment of cell viability showed that all cells were viable after 72 h of contact with the extracts at MOI 0.001. The crude extracts did not show activity against the virus for concentrations under 500.0 µg/ml. After isolation, the polysaccharide fractions still did not show cytotoxic activity. C1p and C4p fractions
exhibited activities against HSV-1 with a respective EC$_{50}$ of 373.0 ± 20.7 µg/ml and
320.9 ± 33.6 µg/ml. The polysaccharides obtained from other extracts did not show
activity in the range of concentration.

The antiviral activity of the samples C1$_p$ and C4$_p$ is correlated to the high amounts of
rhamnose, the main component of ulvans, coupled with a higher purity. The antiviral
activity of sulfated polysaccharides from seaweed had already been shown in previous
studies [6, 30]. However, the EC$_{50}$ are higher than those obtained for other sulfated
polysaccharides.

This work is the first reporting the antiviral activity of enzyme-assisted extracts
containing ulvans from *U. armoricana*. For many years, there has been an increasing
demand from the pharmaceutical industry for the discovery of new antiviral molecules.
The huge *Ulva* sp. biomass might therefore respond to this demand [6].

3.9 DPPH scavenging - Antiradical activity

The free radical scavenging of the blank and the enzymatic extracts of *Ulva armoricana*
was estimated by the decrease in absorbance due to the reduction of the DPPH radical
by the extracts (Table IV). The standards BHA and BHT present an inhibiting
concentration (IC$_{50}$) of 4.8 and 6.8 µg/ml, respectively. Samples obtained with P1, C1
and P2 presented positive results with an IC$_{50}$ of 1.8, 6.0 and 12.5 mg/ml, respectively.
The other samples did not have a significant antiradical effect in the concentration range
used. As previously reported [31], the antioxidant power of the extract is generally
related to the presence of phenolic compounds in seaweed and/or seaweed extracts.
Some studies also reported that sulfated polysaccharides (ulvans) could also have an
antiradical effect [32]. According to the biochemical composition, the three bioactive extracts are also the most concentrated in polyphenols, with respective concentrations of 0.8 %, 0.6 % and 1.0 %. Secondly, no significant differences appear, between these samples and the others, in the ulvan content. Therefore, the antiradical activity could be related to phenolic compounds but some further analyses, before and after specific purification, would need to be realized in order to confirm this hypothesis. Despite its low levels of antioxidant compounds, *Ulva armoricana* could be an interesting and novel bioresource for these compounds. As with antiviral compounds, the pharmaceutical, cosmetic and food industries, which currently use mainly synthetic molecules, are constantly looking for new sources of natural sources of antiradical molecules. Antioxidant compounds are used in order to limit the organoleptic degradation of food, as anti-aging in cosmetics or as drugs in the treatment of oxidative stress [9, 18].

4. Conclusion

This study demonstrates the potential of Enzyme-Assisted Extraction for the valorization of *Ulva* sp. biomass. The improvement of extraction yields using proteases and the selective degradation of β-glucans using carbohydrases have been demonstrated. This is the first study reporting the efficiency of proteases for the liquefaction without degradation of *Ulva armoricana* components. These extraction procedures and the isolation step allowed the preparation of samples enriched in ulvans. Finally, the relationship between ulvans and antiviral activity has been established.


Figure 1: Percent dry weight (% total dw) in extracts after the enzyme-assisted extraction (EAE). The respective enzymatic preparations inputs have been deducted from the dry samples. Values are means ± SD (n=3). Values with different superscript letters are significantly different (p < 0.05). Proteases are indicated with black bars; Carbohydrases by grey bars; Water extract by a white bar.


Figure 2: High pressure steric exclusion chromatogram (RI) of samples C4 and C4p, before and after ethanol precipitation. Flow: 0.5 ml/min; Pressure: 9-13 bars; Eluent: 0.1M NaNO3; Sample: 1 mg/ml; Inject. vol.: 100 µL, detection: refractive index. C4: dotted line; C4p: solid line.
Tables and Figures

Figure 1: Percent dry weight (% total dw) in extracts after the enzyme-assisted extraction (EAE). The respective enzymatic preparations inputs have been deducted from the dry samples. Values are means ± SD (n=3). Values with different superscript letters are significantly different (p < 0.05). Proteases are indicated with black bars; Carbohydrases by grey bars; Water extract by a white bar.

TABLE I: Biochemical composition of raw material and enzymatic extracts (% dw and g).

Values are means ± SD (n=3). Values with different superscript letters are significantly different (p < 0.05). Organic matter was determined by the difference between the total dry matter and the ash content.

<table>
<thead>
<tr>
<th></th>
<th>Ash %dw</th>
<th>Organic matter %dw</th>
<th>Neutral sugars %dw</th>
<th>Uronic acids %dw</th>
<th>Sulfate %dw</th>
<th>Proteins %dw</th>
<th>Total phenols %dw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw algae</td>
<td>15.9 ± 1.2</td>
<td>84.1 ± 1.2</td>
<td>23.2 ± 1.4</td>
<td>21.1 ± 0.1</td>
<td>20.1 ± 0.9</td>
<td>24.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Blank</td>
<td>18.0 ± 1.2a</td>
<td>82.0 ± 1.2a</td>
<td>9.0 ± 1.2a</td>
<td>21.5 ± 4.2a</td>
<td>17.2 ± 0.1d</td>
<td>9.4 ± 0.4ad</td>
<td>0.5 ± 0.0abc</td>
</tr>
<tr>
<td>C1</td>
<td>17.3 ± 1.6a</td>
<td>82.7 ± 1.6a</td>
<td>18.4 ± 0.8d</td>
<td>16.2 ± 0.9d</td>
<td>19.1 ± 0.3c</td>
<td>8.8 ± 0.9c</td>
<td>0.7 ± 0.2a</td>
</tr>
<tr>
<td>C2</td>
<td>17.0 ± 2.7a</td>
<td>83.0 ± 2.7a</td>
<td>8.1 ± 1.6a</td>
<td>10.9 ± 1.3ab</td>
<td>17.4 ± 0.2d</td>
<td>6.2 ± 0.4a</td>
<td>0.6 ± 0.1abc</td>
</tr>
<tr>
<td>C3</td>
<td>17.7 ± 1.8a</td>
<td>82.3 ± 1.8a</td>
<td>18.3 ± 3.3d</td>
<td>19.9 ± 1.2ab</td>
<td>17.4 ± 0.1d</td>
<td>7.3 ± 0.7b</td>
<td>0.4 ± 0.1a</td>
</tr>
<tr>
<td>C4</td>
<td>18.6 ± 2.1a</td>
<td>81.4 ± 2.1a</td>
<td>14.1 ± 0.5bc</td>
<td>13.7 ± 2.3bc</td>
<td>14.3 ± 0.4c</td>
<td>8.5 ± 0.4c</td>
<td>0.7 ± 0.1b</td>
</tr>
<tr>
<td>P1</td>
<td>16.7 ± 2.1a</td>
<td>83.3 ± 2.1a</td>
<td>14.7 ± 0.5c</td>
<td>15.6 ± 0.6c</td>
<td>15.7 ± 0.1c</td>
<td>10.1 ± 0.3d</td>
<td>0.9 ± 0.1a</td>
</tr>
<tr>
<td>P2</td>
<td>16.7 ± 0.2a</td>
<td>83.3 ± 0.2a</td>
<td>11.3 ± 2.3bc</td>
<td>9.6 ± 2.3a</td>
<td>14.8 ± 0.3bc</td>
<td>6.5 ± 0.3abc</td>
<td>1.1 ± 0.2c</td>
</tr>
<tr>
<td>g</td>
<td>6.0 ± 0.1</td>
<td>31.5 ± 0.4</td>
<td>8.7 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>7.5 ± 0.1</td>
<td>9.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>g</td>
<td>3.1 ± 0.3a</td>
<td>13.9 ± 0.2a</td>
<td>1.5 ± 0.3a</td>
<td>3.6 ± 0.7bc</td>
<td>2.9 ± 0.1a</td>
<td>1.6 ± 0.1a</td>
<td>0.1 ± 0.0a</td>
</tr>
<tr>
<td>g</td>
<td>3.0 ± 0.2a</td>
<td>14.4 ± 0.7a</td>
<td>3.2 ± 0.2b</td>
<td>2.8 ± 0.2b</td>
<td>3.3 ± 0.2c</td>
<td>1.5 ± 0.2c</td>
<td>0.1 ± 0.0a</td>
</tr>
<tr>
<td>g</td>
<td>2.8 ± 0.6a</td>
<td>13.9 ± 0.2a</td>
<td>1.4 ± 0.6a</td>
<td>1.8 ± 0.2a</td>
<td>2.9 ± 0.1a</td>
<td>1.0 ± 0.1a</td>
<td>0.1 ± 0.0a</td>
</tr>
<tr>
<td>g</td>
<td>3.1 ± 0.5a</td>
<td>14.5 ± 1.0a</td>
<td>3.2 ± 0.8b</td>
<td>3.5 ± 0.2bc</td>
<td>3.1 ± 0.1a</td>
<td>1.3 ± 0.2b</td>
<td>0.1 ± 0.0a</td>
</tr>
<tr>
<td>g</td>
<td>4.8 ± 0.8ab</td>
<td>21.2 ± 0.4b</td>
<td>3.7 ± 0.2bc</td>
<td>3.5 ± 0.6bc</td>
<td>3.7 ± 0.3d</td>
<td>2.2 ± 0.1d</td>
<td>0.2 ± 0.0b</td>
</tr>
<tr>
<td>g</td>
<td>4.6 ± 0.9bc</td>
<td>23.1 ± 0.3c</td>
<td>4.1 ± 0.2c</td>
<td>4.3 ± 0.8c</td>
<td>4.4 ± 0.1a</td>
<td>2.8 ± 0.1c</td>
<td>0.2 ± 0.0b</td>
</tr>
<tr>
<td>g</td>
<td>5.5 ± 0.1a</td>
<td>27.6 ± 0.1d</td>
<td>3.7 ± 0.8bc</td>
<td>3.6 ± 0.2b</td>
<td>4.9 ± 0.3c</td>
<td>2.1 ± 0.1d</td>
<td>0.4 ± 0.1d</td>
</tr>
</tbody>
</table>
TABLE II: Amino-acids composition of extracts (g/100 g proteins)

Ala: alanine; Asp : aspartic acid; Glu : glutamic acid; Gly: glycine; His : histidine;
Hyp : hydroxyproline; Ile: isoleucine; Leu: leucine; Lys : lysine; Met : methionine;
Phe : phenylalanine; Pro : proline; Ser : serine; Thr : threonine; Tyr : tyrosine; Val:
valine.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>P1</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>16.3</td>
<td>13.6</td>
<td>13.7</td>
<td>14.1</td>
<td>13.0</td>
<td>12.1</td>
<td>12.2</td>
</tr>
<tr>
<td>Asp</td>
<td>11.1</td>
<td>14.5</td>
<td>14.1</td>
<td>14.4</td>
<td>14.0</td>
<td>13.6</td>
<td>12.9</td>
</tr>
<tr>
<td>Glu</td>
<td>8.1</td>
<td>13.2</td>
<td>13.1</td>
<td>14.3</td>
<td>12.8</td>
<td>11.0</td>
<td>9.3</td>
</tr>
<tr>
<td>Gly</td>
<td>11.1</td>
<td>9.0</td>
<td>9.0</td>
<td>8.9</td>
<td>8.7</td>
<td>8.7</td>
<td>9.1</td>
</tr>
<tr>
<td>His</td>
<td>1.1</td>
<td>0.9</td>
<td>1.1</td>
<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Hyp</td>
<td>2.3</td>
<td>2.3</td>
<td>2.1</td>
<td>2.0</td>
<td>1.7</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Ile</td>
<td>5.2</td>
<td>5.2</td>
<td>5.3</td>
<td>5.1</td>
<td>5.3</td>
<td>5.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Leu</td>
<td>8.4</td>
<td>7.1</td>
<td>7.3</td>
<td>7.0</td>
<td>7.6</td>
<td>8.3</td>
<td>8.8</td>
</tr>
<tr>
<td>Lys</td>
<td>2.6</td>
<td>3.0</td>
<td>3.2</td>
<td>3.1</td>
<td>2.9</td>
<td>2.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Met</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Phe</td>
<td>4.9</td>
<td>4.3</td>
<td>4.0</td>
<td>3.9</td>
<td>4.6</td>
<td>5.5</td>
<td>6.3</td>
</tr>
<tr>
<td>Pro</td>
<td>6.6</td>
<td>5.9</td>
<td>6.3</td>
<td>6.0</td>
<td>6.3</td>
<td>6.7</td>
<td>6.9</td>
</tr>
<tr>
<td>Ser</td>
<td>5.6</td>
<td>5.4</td>
<td>5.1</td>
<td>4.8</td>
<td>5.4</td>
<td>6.4</td>
<td>6.8</td>
</tr>
<tr>
<td>Thr</td>
<td>6.9</td>
<td>6.4</td>
<td>6.3</td>
<td>6.0</td>
<td>6.7</td>
<td>7.2</td>
<td>7.4</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.5</td>
<td>1.2</td>
<td>1.3</td>
<td>1.2</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Val</td>
<td>7.4</td>
<td>7.1</td>
<td>7.3</td>
<td>7.2</td>
<td>7.3</td>
<td>7.2</td>
<td>7.0</td>
</tr>
</tbody>
</table>
TABLE III: Simple sugars composition of extracts (a) and isolated polysaccharide fractions (b) (g/100g total sugars).

Rha: rhamnose; Gal: galactose; Glc: glucose; Xyl: xylose; UAc: Uronic Acids. ‘Other sugars’ represents the sum of low concentrated and non-identified sugars.

<table>
<thead>
<tr>
<th></th>
<th>Rha</th>
<th>Gal</th>
<th>Glc</th>
<th>Xyl</th>
<th>UAc</th>
<th>Other Sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blk</td>
<td>40.0</td>
<td>6.7</td>
<td>26.2</td>
<td>4.4</td>
<td>13.6</td>
<td>9.2</td>
</tr>
<tr>
<td>C1</td>
<td>33.5</td>
<td>5.0</td>
<td>36.4</td>
<td>4.5</td>
<td>11.3</td>
<td>9.3</td>
</tr>
<tr>
<td>C2</td>
<td>27.2</td>
<td>3.8</td>
<td>32.5</td>
<td>4.0</td>
<td>8.8</td>
<td>23.5</td>
</tr>
<tr>
<td>C3</td>
<td>26.9</td>
<td>3.6</td>
<td>36.4</td>
<td>3.8</td>
<td>9.2</td>
<td>20.1</td>
</tr>
<tr>
<td>C4</td>
<td>26.1</td>
<td>3.9</td>
<td>48.8</td>
<td>2.5</td>
<td>10.1</td>
<td>8.6</td>
</tr>
<tr>
<td>P1</td>
<td>30.6</td>
<td>3.9</td>
<td>28.1</td>
<td>2.8</td>
<td>11.9</td>
<td>22.6</td>
</tr>
<tr>
<td>P2</td>
<td>35.1</td>
<td>4.3</td>
<td>31.7</td>
<td>4.2</td>
<td>14.1</td>
<td>10.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Rha</th>
<th>Gal</th>
<th>Glc</th>
<th>Xyl</th>
<th>UAc</th>
<th>Other Sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blk</td>
<td>45.8</td>
<td>4.3</td>
<td>23.6</td>
<td>5.4</td>
<td>12.6</td>
<td>8.2</td>
</tr>
<tr>
<td>C1</td>
<td>53.7</td>
<td>4.4</td>
<td>15.5</td>
<td>4.1</td>
<td>13.4</td>
<td>8.7</td>
</tr>
<tr>
<td>C2</td>
<td>53.4</td>
<td>5.1</td>
<td>9.2</td>
<td>4.3</td>
<td>16.5</td>
<td>11.5</td>
</tr>
<tr>
<td>C3</td>
<td>55.6</td>
<td>4.4</td>
<td>11.4</td>
<td>3.7</td>
<td>13.7</td>
<td>11.3</td>
</tr>
<tr>
<td>C4</td>
<td>42.3</td>
<td>4.7</td>
<td>26.0</td>
<td>5.4</td>
<td>13.1</td>
<td>8.5</td>
</tr>
<tr>
<td>P1</td>
<td>41.9</td>
<td>3.9</td>
<td>26.2</td>
<td>5.1</td>
<td>13.1</td>
<td>9.7</td>
</tr>
<tr>
<td>P2</td>
<td>47.9</td>
<td>4.5</td>
<td>20.8</td>
<td>4.6</td>
<td>13.6</td>
<td>8.7</td>
</tr>
</tbody>
</table>
Figure 2: High pressure steric exclusion chromatogram (RI) of samples C4 and C$_{4p}$, before and after ethanol precipitation.

Flow: 0.5 ml/min; Pressure: 9-13 bars; Eluent: 0.1M NaNO$_3$; Sample: 1 mg/ml; Inject. vol.: 100 µL, detection: refractive index. C4: dotted line; C$_{4p}$: solid line.
TABLE IV: Screening of *in vitro* cytotoxic, antiviral and antioxidant activities of crudes extracts and polysaccharides fractions.

* BHA : Butylated HydroxyAnisole; ** BHT : Butylated HydroxyToluene

<table>
<thead>
<tr>
<th></th>
<th>Crude extracts</th>
<th>Polysaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytotoxicity</td>
<td>Antiviral</td>
</tr>
<tr>
<td></td>
<td>$CC_{50}$ ($\mu$g/ml)</td>
<td>$EC_{50}$ ($\mu$g/ml)</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>&gt; 500.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>BHA$^*$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BHT$^{**}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blk</td>
<td>&gt; 500.0</td>
<td>&gt; 500.0</td>
</tr>
<tr>
<td>C1</td>
<td>6.0</td>
<td>C1$_p$</td>
</tr>
<tr>
<td>C2</td>
<td>&gt; 500.0</td>
<td>&gt; 500.0</td>
</tr>
<tr>
<td>C3</td>
<td>&gt; 500.0</td>
<td>&gt; 500.0</td>
</tr>
<tr>
<td>C4</td>
<td>&gt; 500.0</td>
<td>&gt; 500.0</td>
</tr>
<tr>
<td>P1</td>
<td>1.8</td>
<td>P1$_p$</td>
</tr>
<tr>
<td>P2</td>
<td>12.5</td>
<td>P2$_p$</td>
</tr>
</tbody>
</table>