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Response Surface Methodology for Enzyme-Assisted Extraction of Water-Soluble Antiviral Compounds from the Proliferative Macroalga *Solieria chordalis*

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Abstract

Macroalgal blooms frequently occur in France. On a part of the coastline, these algal blooms are mainly composed of red seaweeds like *Solieria chordalis* and constitute an unexploited significant natural biomass. In this study, active compounds from *Solieria chordalis* were extracted and evaluated as a potential source of natural antivirals, coupling biotechnological development with economic and ecological benefits. In order to extract in water the highest quantity of potential active compounds, a sustainable process was developed, namely the enzyme-assisted extraction. The quantity of water-soluble compounds increased by 30% after the addition of enzymes, in comparison with an aqueous extraction. The optimization of conditions using a response surface methodology improved the yield and allowed to study the influence of different extraction parameters simultaneously, notably the nature and the quantity of enzymes, the temperature and the time of extraction. This latter parameter was the most influent on extraction yield with the nature of the enzyme. The best antiherpetic activity was obtained with the extract after the action of a type of proteases with an EC₅₀ of 86.0 µg.mL⁻¹. Moreover, a positive correlation between sulfated polysaccharides and the antiviral activity of extracts was demonstrated. For the first time, soft biotechnology with enzymes using surface response methodology has been performed in order to obtain water-soluble antiviral extracts from the red proliferative seaweed *Solieria chordalis*.

Keywords: Antibiotics; Bacteria; Popular knowledge; Metabolism; Natural products

Abbreviations: EAE: Enzyme-Assisted Extraction; HSV-1: Herpes Simplex Virus Type 1; WSE: Water-Soluble Extract.

Introduction

Excessive growths reported for some species of green, brown and red seaweeds are responsible for the recent formation of **harmful** marine macroalgal blooms worldwide. Local wind and tides drive seaweeds to the shore, causing the destruction of coastal marine habitats and economical losses. Each year, in South Brittany, in France, over 2,000 tons of red seaweeds, mainly composed of *Solieria chordalis*, have to be removed [1].

Largely unexploited, *S. chordalis* holds considerable potential for biotechnological development, since some of its polymers have already been shown to possess immunological, haemagglutinin, and antiviral activities with practical application in biomedicine [2,3].

In addition to the compounds inside seaweed cells, there are other potential active components associated with the cell walls [4]. To isolate these metabolites of interest, the cell wall must be cleaved, but the presence of large quantities of various interconnected polysaccharides and their bonding with proteins reduces the efficiency of the standard extraction methods used to date (e.g. organic and water extractions) [5]. Enzyme-assisted extraction (EAE) is an environmentally friendly extraction method known by its high efficiency and reduced solvent consumption and time [4]. In this regard, proteins, especially enzymes from marine and terrestrial microorganisms, are used to release in water the maximum number of compounds from seaweeds, thus improving their availability and their digestibility for animals, humans, or plants [5,6].

Numerous studies have shown the benefits of using enzymes to add value to seaweed extracts or compounds [3,4,7], but only a few deal with the optimization of hydrolysis conditions on algal material

[5]. Parameters such as the time needed for the enzymatic hydrolysis, temperature, nature and concentration of enzymes, pH, and raw material pretreatment have shown their impact on the extraction [8]. Response surface methodology is based on the fit of statistical models to measured data obtained in relation to an experimental design. This methodology has been shown to be useful for improving, developing, and optimizing biochemical processes to extract the maximum number of compounds of interest [8]. The Box-Behnken design was chosen to investigate the influence of three parameters on the extraction yield of water-soluble compounds, notably the time of extraction, the temperature, and the enzyme quantity used on dried seaweeds.

In this study, the isolation of antiviral extracts from the red macroalga *Solieria chordalis* was performed by using enzymes. After selecting the most efficient enzyme in the release of antiviral compounds, an experimental design procedure was used to increase the extraction yield.

Materials and Methods

Algal material

About 20 kg of *S. chordalis* (Rhodophyta, Gigartinales) were collected in October 2013 from the littoral zone of Saint Gildas de Rhuy in Brittany, France. Seaweeds were washed with tap water. They

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were scraped and drained to remove adherent seawater, sediment and epiphytes. Cleaned and wrung out seaweeds were ground to pieces of 3 mm with a hammer mill prior to freeze drying.

Enzymes

Eight commercial enzyme solutions from Novozymes (Le Pecq, France) were separately evaluated for the extraction of *S. chordalis*: three proteases (subtilisin, neutral metallo-proteinase and exopeptidase) that are named P1 to P3 and five carbohydrases (amyloglucosidase, alpha-amylase, endo-xylanase, endo-beta-glucanase, polygalacturonase beta-glucanase) that are named C1 to C5 respectively.

Cells and virus

African green monkey kidney cells (ATCC CCL-81) were grown in Eagle's minimum essential medium (MEM, Eurobio, Courtaboeuf, France) supplemented with 8% fetal calf serum (FCS, Eurobio) and 1% of antibiotics (10,000 IU mL⁻¹ penicillin, 25,000 IU mL⁻¹ colimycin, 10 mg.mL⁻¹ streptomycin, Sigma Aldrich).

HSV-1 (*Herpes Simplex Virus* type 1, wild type strain 17) was obtained from Pr. Agut, Laboratoire de Dynamique, épidémiologie et traitement des infections virales in Pitié Salpêtrière (Paris, France). Viruses are stored in infected Vero cells bathing in supplemented MEM at -80°C before use.

Extractions

To characterize the raw matter of *S. chordalis*, 10 mg of freeze-dried matter were mixed with 5 mL HCl 1M in a sealed vial. The acid extraction was performed at 100°C for 2 h, after which 5 mL NaOH 1M were added. The final solution was used to measure the neutral sugars, uronic acids, and proteins contents. To analyze the sulfate groups linked to polysaccharides, the same conditions of extraction were performed in ultrapure water.

EAE was achieved in a final volume of 300 mL with 19.5 g of freeze-dried matter of *S. chordalis* and the rest with distilled water. Enzymes (5% dw/dw) were added to the mixture that is incubated at 50°C. Extractions were carried out for 3 hours. Enzymes were then denatured at 85°C for 15 min. The obtained water-soluble extracts (WSE) were filtered, separated from the washed residue and freeze-dried. A blank extracted in the same conditions (3h, 50°C) but without enzymes served as a control. Each extraction was performed in triplicate.

Extraction yield determination

The EAE yield represents the proportion, in percentage, of the dry weight of *S. chordalis* found in the WSE. The dry weight of WSE was determined gravimetrically after incubation at 100°C overnight.

Biochemical composition analysis

The composition of *S. chordalis* and of WSE is defined as the proportions in percentage of each chemical compound family found in the total dried weight of the raw matter or of WSEs. All the biochemical analyses were carried out in triplicate. Neutral sugars were determined by the phenol sulphuric acid method described by Dubois et al. [9]. Uronic acids were determined by using the meta-hydroxy-di-phenyl (MHDP) method [10]. Sulfated groups content was determined by the Azure A method that reacts specifically with sulfates linked to the polysaccharides [11]. Lipids were extracted with a mixture of chloroform/methanol (1:1, v/v) over 2 days under agitation. The extract was filtered and washed with distilled water. The lipid content was determined gravimetrically. Proteins were quantified by the

bicinchoninic acid colorimetric method (BCA) with a Micro BC Assay Kit (Interchim, Montluçon, France) [12]. Ash values were determined gravimetrically after incineration of samples followed by 2 h at 700°C.

Cytotoxicity and antiherpetic activity

Dilutions of samples (1 µg/mL to 200 µg/mL) were prepared in Eagle's MEM supplemented with 8% FCS and distributed into a 96-well plate. One hundred microliters of cellular suspension (3.5 × 10⁵ Vero mammalian cells/mL) in supplemented Eagle's MEM were added in each well. In half of the microplate, cells were infected by the HSV-1 (*Herpes Simplex Virus* type 1) at a multiplicity of infection of 0.001 ID₅₀/cells. The last two columns of the 96-well plate were used for the controls of the living cells and infected cells by HSV-1. The 96-well plate was incubated for 3 days at 37°C with 5% CO₂.

Cytotoxicity was tested using cell viability by the neutral red dye method. Optical density (OD) was measured at 540 nm. The 50% cytotoxic concentration (CC₅₀) was defined as the concentration of seaweed extract that reduced the OD of treated cells to 50% of that of untreated cells [13].

The antiherpetic compound acyclovir was used as reference inhibitor. The 50% effective antiviral concentration (EC₅₀) was expressed as the concentration that achieved 50% protection of virus-infected cells [13].

Experimental design for the optimization

Three parameters were examined: the time of the extraction, the temperature, and the ratio of enzyme on substrate (E/S). They were varied into three levels. The measured response was the extraction yield of water soluble compounds. A total of 13 experiments were performed in triplicate (Table 1). The order of the experiments was fully randomized. To check the model predicted by the statistical analyses, experiments were conducted (Table 1).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). The statistical analyses were carried out with the software Minitab[®] 17 using one-way analysis of variance (ANOVA) according to the Fisher's Least Significant Difference (LSD) test at 5% level to evaluate the differences between extracts or extraction parameters.

Results

Biochemical composition of *S. chordalis*

The biochemical composition of the red macroalga *S. chordalis* is given in Table 2.

S. chordalis is composed essentially of water up to 90%. The dry matter is rich in ash (43.3%) and in sulfated polysaccharides (a little less than 49.5% if the neutral sugars, sulfate groups, and uronic acids contents are gathered and taking in account interferences between compounds during the biochemical assays). Lipids represented a small percentage of the dry weight of the macroalgae (3.0%). Proteins constituted over one fifth of the dry matter (22%).

Screening of enzymes

The effect of the different enzymes on the extraction yield is shown in Figure 1.

All WSEs, after the action of enzymes, contain quantitatively more compounds than the blank. The protease P1 is significantly the most

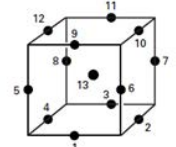
	Levels of independent variables used	Extraction time (min)	Temperature (°C)	E/S (% DW algae)
	-1	90	25	2.5
	0	180	37.5	5
	1	270	50	7.5

Table 1: Box-Behnken experimental design and levels of independent variables used to optimize the enzyme-assisted extraction

Dry weight	Ash content	Proteins	Lipids	Uronic acids	Sulfate groups	Neutral sugars
(% FW) ^a	(% DW) ^b	(% DW) ^b	(% DW) ^b	(% DW) ^b	(% DW) ^b	(% DW) ^b
10.0 ± 0.6	43.3 ± 3.1	22.0 ± 2.0	3.0 ± 0.1	3.1 ± 0.1	14.7 ± 1.0	31.7 ± 0.7

^a Percentage of dry weight (DW) in the fresh weight (FW).

^b Calculated on the dry weight.

Values are means ± SD (n=9).

Table 2: Dry weight and biochemical composition of *S. chordalis*.

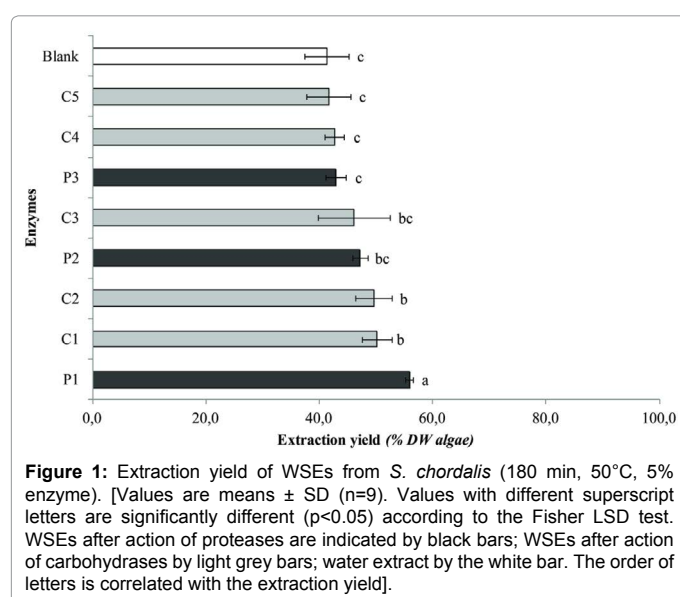


Figure 1: Extraction yield of WSEs from *S. chordalis* (180 min, 50°C, 5% enzyme). [Values are means ± SD (n=9). Values with different superscript letters are significantly different (p<0.05) according to the Fisher LSD test. WSEs after action of proteases are indicated by black bars; WSEs after action of carbohydrases by light grey bars; water extract by the white bar. The order of letters is correlated with the extraction yield].

efficient in the release of water-soluble compounds as the extraction yield reached almost 60% (55.8%). The gain of the released compounds found in the WSE due to the action of P1 is 30%, compared to an aqueous extraction in the same conditions (Blank). Extraction yields after the action of carbohydrases C1 to C3 and of the protease P2 are not significantly different and are between 46.1 and 50.1%, but only C1 and C2 increased significantly the yield compared to the blank. The others enzymes (C4, C5, and P3) and the blank obtained a similar yield between 41.3 and 43.0% of the total compounds present in *S. chordalis*.

Biochemical compositions of extracts

The proportions of water-soluble compounds composing the dry weight of WSEs are presented in Table 3. To measure the rates of the different compounds families, no acid or water extraction was performed. Only the available and free compounds of the extracts were then considered. In the last column titled *Others*, no identified compounds are found. They may correspond to agglomerates, or other compounds that are not detected by the biochemical analyses used. All enzyme-assisted extractions solubilized an important proportion of ash from 55.0 to 64.2%. Nevertheless, more organic matter was extracted due to the action of enzymes. Less than 20% organic matter was found in the blank and up to 45% after the action of enzymes. In the soluble extracts, between 9.1 and 33.3% of neutral sugars were

obtained, whereas only 4.5 to 8.9% of proteins were present. Few sulfate groups (1.6 to 4.2%) as well as uronic acids (0.2 to 1.3%) were detected. The proteases P1 and P2 allowed to access to the highest proportions of proteins (8.9 and 8.8% of the dry matter) and the carbohydrase C5 to the highest proportion of neutral sugars (33.3%) followed by P1 (27.5%) (Table 3).

Cytotoxicity and antiviral activity

After 3 days of viral infection, visible alteration of normal cell morphology was observed under the microscope except for the cells in contact with seaweed extracts. No cytotoxic effect on Vero cells was detected for all the WSEs for a CC₅₀ inferior to 200 µg.mL⁻¹ (Table 4). The lowest EC₅₀ that represents the most efficient antiviral activity was obtained with the P1 extract (86.0 µg.mL⁻¹) followed by the extracts obtained with P3 and with carbohydrases (C1, C2, C5 and C3 respectively). The P2 extract showed the least efficient antiherpetic activity with an EC₅₀ of 145.9 µg.mL⁻¹ and was not significantly different than those measured in the blank (170.7 µg.mL⁻¹) (Table 4).

Optimization by Box-Behnken design

The protease P1 was selected for optimizing extraction conditions in order to increase potentially the active compounds quantity. The responses that correspond to extraction yields following different conditions of enzymatic digestion are reported in (Table 5).

Three enzyme-assisted extraction conditions significantly resulted in a similar quantity of compounds in extracts (Experiments N°3, 10 and 11) with a maximum extraction yield of 59.4% (N°11). Compared to the screening conditions with P1, the yield increased by 6.5% with 7.5% enzymes on dry matter of *S. chordalis* during the same time (180 min) and at the same temperature (50°C). An extraction time from 180 min to 270 min was necessary to reach a maximum number of water soluble compounds. Moreover, 90 min were not sufficient to break down the cell walls, regardless of the other examined parameters. The highest tested temperature (50°C) enabled the extraction of a maximum number of compounds during 180 min. Nevertheless, at 25 and 37.5°C, relatively good extraction yields were measured (52.4 to 56.5%). Concerning the relation E/S, for two of the best conditions, extractions assisted with 7.5% of P1 were the most efficient.

Predicted optimal extraction conditions

According to the response obtained for each experiment (Table 5), the best explanatory model equation for the enzyme-assisted extraction yield (EAE %) with *T*, the temperature (°C), *t*, the time (min) and *E/S* the enzyme on substrate ratio (%):

	Neutral sugars (%DW)	Sulfate groups (%DW)	Uronic acids (%DW)	Proteins (%DW)	Ash (%DW)	Others (%DW)
Blank	^g 12.8 ± 0.1	^f 1.6 ± 0.4	^b 1.0 ± 0.1	^d 4.5 ± 0.1	^a 70.9 ± 1.0	^b 9.2 ± 1.7
C1	^d 22.8 ± 0.9	^{ab} 3.7 ± 0.2	^c 0.6 ± 0.1	^{abc} 8.4 ± 0.6	^b 64.2 ± 0.4	^c 0.3 ± 2.1
C2	^{cd} 24.1 ± 1.3	^{cd} 2.7 ± 0.3	^b 1.0 ± 0.1	^{abc} 7.8 ± 0.8	^d 55.0 ± 0.9	^b 9.4 ± 3.4
C3	^e 19.9 ± 0.8	^b 3.4 ± 0.3	^d 0.2 ± 0.1	^c 7.1 ± 0.1	^b 64.0 ± 4.4	^{bc} 5.4 ± 5.7
C4	^h 9.1 ± 1.5	^{de} 2.3 ± 0.3	^b 1.1 ± 0.1	^d 5.3 ± 0.2	^b 64.0 ± 4.6	^a 18.3 ± 6.7
C5	^a 33.3 ± 0.8	^b 3.3 ± 0.1	^d 0.2 ± 0.1	^{ab} 8.5 ± 0.7	^{bcd} 58.9 ± 2.4	^c 0.1 ± 4.1
P1	^b 27.5 ± 0.3	^{bc} 3.2 ± 0.1	^d 0.3 ± 0.2	^a 8.9 ± 2.0	^{cd} 56.5 ± 1.6	^{bc} 3.6 ± 4.2
P2	^f 17.6 ± 0.8	^{ef} 1.9 ± 0.2	^a 1.3 ± 0.1	^a 8.8 ± 0.4	^{bc} 61.2 ± 6.6	^b 9.3 ± 8.1
P3	^c 25.4 ± 1.2	^a 4.2 ± 0.5	^c 0.5 ± 0.1	^{bc} 7.3 ± 0.3	^b 63.9 ± 0.5	^c 0.1 ± 2.6

Values are means ± SD (n=9). For each column, values with different superscript letters are significantly different (p<0.05) according to the Fisher LSD test. The order of letters is correlated with the compounds contents.

Table 3: Biochemical composition of extracts after the action of different enzymes (180 min, 50°C, 5% enzyme).

	CC ₅₀ (µg / mL)	EC ₅₀ (µg / mL)
P1	>200 ± 0.9	^a 86.0 ± 9.1
P3	>200 ± 0.8	^a 96.7 ± 4.7
C1	>200 ± 3.3	^a 98.6 ± 7.1
C2	>200 ± 1.0	^{ab} 109.8 ± 12.1
C5	>200 ± 2.4	^{ab} 110.0 ± 6.5
C3	>200 ± 0.1	^{ab} 113.7 ± 19.1
C4	>200 ± 2.4	^{bc} 136.2 ± 46.3
P2	>200 ± 9.6	^{cd} 145.9 ± 12.2
Blank	>200 ± 1.3	^d 170.7 ± 11.9
Drug (acyclovir)	>200 ± 0.2	^d 0.5 ± 0.4

MOI: multiplicity of infection of 0.001 ID₅₀ / cells: ratio (virus titer) / (cells numbers mL⁻¹).
 CC₅₀: the 50% cytotoxic concentration. The concentration that reduced the absorbance of mock-infected cells to 50% of that of control.
 EC₅₀: the 50% antiviral effective concentration: concentration that achieved 50% protection of virus-infected cells from the HSV-induced destruction.
 Values are means ± SD (n=8).
 Values with different superscript letters are significantly different (p<0.05) according to the Fisher LSD test. The order of letters is correlated with the efficiency of activity

Table 4: Evaluation of anti-HSV-1 activity on Vero cell line of WSEs from *S. chordalis*, by neutral red dye method.

Experiment N°	Hydrolysis conditions			Obtained response EAE yield extraction (% dw algae)
	Extraction time (min)	Temperature (°C)	E/S (% dw algae)	
1	180	25	2.5	^{bc} 53.9 ± 1.9
2	270	37.5	2.5	^{cde} 51.7 ± 2.5
3	180	50	2.5	^{ab} 57.5 ± 2.1
4	90	37.5	2.5	^f 35.6 ± 4.0
5	90	25	5	^e 46.1 ± 6.8
6	270	25	5	^{cde} 49.6 ± 1.6
7	90	50	5	^{de} 48.4 ± 2.9
8	270	50	5	^{de} 48.0 ± 2.8
9	180	25	7.5	^{bc} 54.0 ± 1.4
10	270	37.5	7.5	^{ab} 56.5 ± 2.5
11	180	50	7.5	^a 59.4 ± 1.6
12	90	37.5	7.5	^f 40.4 ± 1.7
13	180	37.5	5	^{bcd} 52.4 ± 3.0

Values are means ± SD (n=9). Values with different superscript letters are significantly different (p<0.05) according to the Fisher LSD test. The order of letters is correlated with the extraction yield.

Table 5: EAE conditions and responses for the extraction yield of water-soluble compounds from *S. chordalis* after the application of experimental design with P1.

$$\text{EAE \%} = 34.4 - 1.202 \times T + 0.405 \times t - 1.42 \times (E/S) + 0.02 \times T^2 - 0.001 \times t^2 + 0.145 \times (E/S)^2 - 0.001 \times T \times t + 0.014 \times T \times (E/S) + 0.001 \times t \times (E/S)$$

This model was generated using the regression coefficients with statistical significance up to 5% probability level. The statistical results for the water-soluble compounds extraction showed a coefficient of determination value of $R^2 = 0.69$ that indicated that the model as fitted can explain almost 70% of the enzyme-assisted extraction yield variability. The model predicted optimal extraction conditions with the desirability of 96.6% for a maximum yield of 59.7% and for an extraction performed at 50°C, for 200 min and with 7.5 % of P1 on dry matter of *S. chordalis*.

Three-dimensional graphs showing the effect of time, ratio E/S, and temperature are illustrated in Figure 2. For each graph, one of the three optimal parameters was fixed.

After around 200 min, the extraction yield does not seem to increase anymore. According to the results in Table 5 and in Figure 2, the extraction time played a dominant role in the process to increase the number of water-soluble compounds. The influence of the relation E/S and the temperature of extraction were insignificant within the experimental range (data not shown).

Validation for a better extraction yield

The results after the EAE under optimal conditions are reported in Table 6. Under such conditions, for a 200 min extraction at 50°C with an enzyme/substrate ratio of 7.5%, the extraction yield reached 57.9%, which is among the highest values compared to those obtained with the experimental design (Table 5). Moreover, this extraction yield is between the lower and upper limits (54.8-64.6%). Thus, the statistical model was checked and validated.

The optimization of extraction conditions allowed an insignificant gain of 4%. The values obtained throughout the whole study for focused responses, i.e., the extraction yield, the biochemical composition of extracts, and their antiviral activity, are summarized in Table 7.

Concerning the quality of extracts obtained, namely their biochemical composition, the extract after the action of P1 and after the optimization of extraction conditions is richer in organic matter especially in proteins (14.7 %) and less in mineral matter (ash). Furthermore, the antiviral activity is kept.

Discussion

Composition of *S. chordalis*

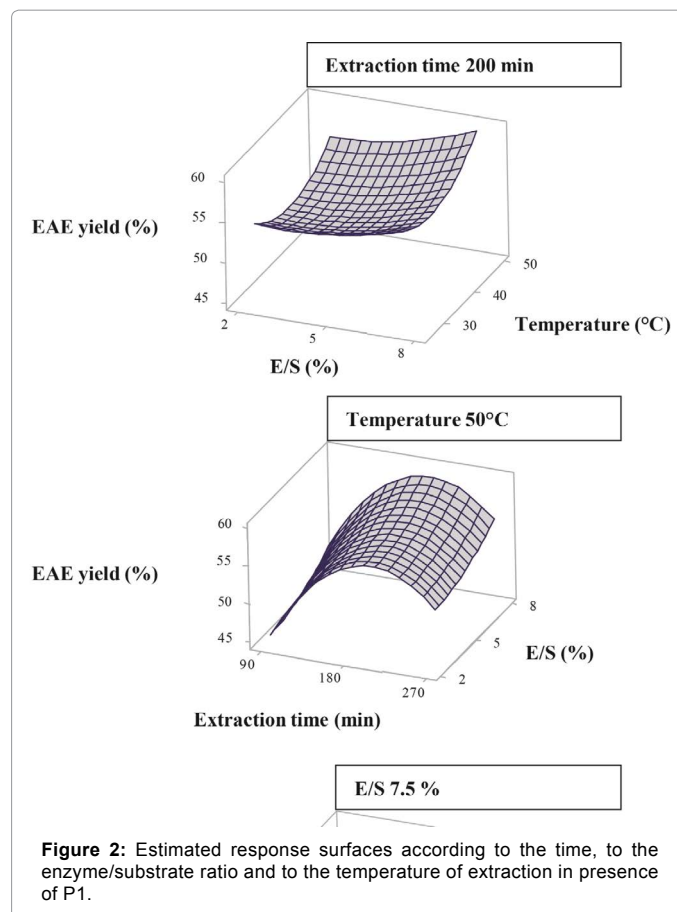
The composition of the dry weight of *S. chordalis* is in agreement with previous studies, but only little information is known. Bondu et al. find a dry matter rate of 14.6% and Hardouin et al. 11.6% for the same species [2,3]. Compared to this previous study, ash content was higher in our study (43.3%). Fast-growing algae, responsible for seaweed blooms, such as *Ulva sp.*, *Gracilaria sp.* and *S. chordalis*, reflect the abundance of nitrogen and other non-organic elements [14]. These chemical elements are naturally abundant and can be found more readily on eutrophic coasts, depending on fluctuating environmental factors and human activities. Polysaccharides represent a high proportion of the dry weight of many macroalgae. In *Porphyra sp.*, about 46% of the dry matter is constituted by carbohydrates, as well as in *Palmaria palmata* (45 to 74% of the dry weight) [15,16]. The percentage concentrations of neutral sugars, uronic acids, sulfate groups, and protein contents in the dry weight of *Solieria chordalis* were

in line with results of the previous study on the same seaweed [3] and with the study of Khanzada et al. [17], who find a percentage between 25 and 32% of proteins in *Solieria robusta* collected in Pakistan. The presence of uronic acids in polysaccharides isolated from Rhodophyta has been reported in a few cases [18].

Solubilization of algal compounds

Enzymes produced from microorganisms can be used to digest seaweeds by breaking down cell walls that release the cell content in water [4,6]. The strategy employed in this study was first to screen different commercial solutions of enzymes with different activities on the same substrate and in identical conditions (50°C, 180 min and 5% E/S). To compare the effect of these enzymes, expected responses were focused on the extraction yield, the biochemical composition of extracts, and their antiviral activity.

Similar conditions (50°C, 300 min and 5% E/S) were applied to *S. chordalis* in a previous study [3]. In our study, the extraction yield of the blank reaches 40% while Hardouin et al. [3] found around 15%. They tested six enzymes, two proteases, and four carbohydrases, and their extraction yields ranged between 15 and 25% of dry matter. Only two parameters varied between the two studies: the time of extraction and the month of the seaweed collection. Shen et al. conducted studies on tea protein extraction using enzyme methods, and they optimized extraction conditions, notably by testing the time. They showed that this parameter has a positive role. Nevertheless, after 4–8 hours, the effect of extraction time became less significant [19]. Moreover, the biochemical composition of raw material is an important parameter to



Response	Prediction	Lower limit (95 %)	Upper limit (95 %)	Value obtained after experiment
EAE yield (% dw)	59.7	54.8	64.6	57.9 ± 2.0

Table 6: Values predicted statically according to optimal conditions and values obtained after experiments.

		Control	Enzyme-assisted extraction	
		Blank	Without optimization	After optimization
Temperature (°C)		50	50	50
Hydrolysis time (min)		180	180	200
E/S ratio (%)		0	5	7.5
Extraction yield (% dw of algae in soluble extract)		^b 43.1 ± 4.5	^a 55.8 ± 0.8	^a 57.9 ± 2
Biochemical composition (% dw of soluble extract)	Neutral sugars	^b 12.8 ± 0.1	^a 27.5 ± 0.3	^b 13.1 ± 0.8
	Uronic acids	^a 1.1 ± 0.1	^a 0.3 ± 0.2	^a 1.3 ± 0.1
	Sulfate groups	^b 1.6 ± 0.4	^a 3.2 ± 0.1	^b 1.7 ± 0.3
	Proteins	^c 4.5 ± 0.4	^b 8.9 ± 2.0	^a 14.7 ± 2.8
	Ash	^a 70.9 ± 1.0	^b 56.5 ± 1.6	^c 51.3 ± 2.2
	Others	^b 9.2 ± 1.7	^b 3.6 ± 4.2	^a 17.9 ± 6.2
Anti-HSV activity (EC ₅₀ in µg/mL)		^b 170.7 ± 11.9	^a 86.0 ± 9.1	^b 163.9 ± 2.8

For each line, values with different superscript letters are significantly different (p<0.05) according to the Fisher LSD test. The order of letters is correlated with the quantity of compounds or with the efficiency of antiviral activity.

Table 7: Summary of EAE conditions and results for water-soluble compounds extraction yield, biochemical composition of extracts and their antiviral activity throughout the optimization process with P1.

consider for extraction. Hardouin et al. [3] found more neutral sugars (around 10% more) than in the present study. Various polysaccharides are found in large quantities, and their bonding with glycoproteins in the cell wall reduces the efficiency of water extractions [5]. The quantity of neutral sugars contributing to the base of polysaccharides slows down the extraction and the action of enzymes.

The nature of components of the seaweed cell wall is well known, contrary to their organization in three dimensions. Proteases seem to be the most efficient in the release of compounds. In our study, the maximum extraction yield reached up to 60% with the protease P1 (Figure 2). P1 is a subtilisin protease. Cleavage by subtilases or serine proteases may be an essential step in breaking down the cell wall of seaweeds by a selective degradation of structural proteins. The red macroalgae possess the most important protein content compared to the other groups (green and brown seaweeds). In *Palmaria palmata* and *Porphyra tenera*, 8 to 47% of proteins are found in the dry matter. Among these proteins, between 2.9 and 6.2% of total amino acids are represented by serine [20]. Serine and threonine may participate in a peptide-O-glycosidic linkage of the extracellular mucilage of red seaweeds. The serine protease P1 probably attacks the serine-glycoproteins of the cell wall. Glycoproteins with serine residue would play a fundamental role in the structure and in the organization in three-dimensions of the red seaweed cell wall. More studies on the red seaweed cell wall need to be conducted to understand and to improve the action of applied enzymes.

Compounds of the cell wall and of the intracellular medium are then released. Soluble, free and active compounds in water are an advantage for applications of the extracts obtained. The use of enzymes improves the availability and the digestibility of seaweed compounds in a water-soluble extract [5]. According to the nature, the specificity, and the selectivity of enzymes, the biochemical compositions of extracts are different. In line with previous studies, the use of enzymatic liquefaction of red seaweeds could be a useful alternative to enhance the sulfated saccharides and the protein solubilization in mild conditions that are useful for diverse applications [4,6,21].

Release of antiviral compounds

A Herpes Simplex Virus type 1 (HSV-1), a DNA enveloped virus, is widely spread in the world. It infects between 60 and up to 95% of certain populations and may cause various illnesses and life-threatening diseases mainly in immune-suppressed individuals such as transplant recipients and cancer and AIDS patients [22]. Sulfated polysaccharides like carrageenans are known to exhibit a viral inhibitory activity [21]. Our results (Table 4) support this affirmation. A positive correlation between the neutral sugar content and the antiviral activity is perceptible. Soluble extracts obtained with P1, P3, C1, C2, C3, and C5 were anti-HSV active with no significant difference in their EC₅₀ (86 to 113.7 µg/mL). These extracts contain more than 23% of sulfated polysaccharides in their dry matter, contrary to the blank and to the extracts after the actions of P2 and C4. Additionally, before the evaluation of antiviral activity, the cytotoxicity of extracts on Vero cells was examined, using the neutral red incorporation with a view to detecting lysosomal functionality. All extracts were well tolerated by Vero mammalian cells. Kulshreshtha et al. [7] have shown an antiviral activity with an enzymatic extract of *Chondrus crispus* with an EC₅₀ of 161.1 µg/mL, and Hardouin et al. [3] found a EC₅₀ between 23 to 101.1 µg/mL with soluble extracts of *S. chordalis*. All these antiherpetic activities obtained with red seaweed extracts are less efficient than the reference molecule Acyclovir (ACV). However, the occurrence of ACV-resistant mutants highlights the necessity to research for new antiviral compounds [22]. The anti-HSV-1 activity of extracts can be enhanced by purifying water-soluble sulfated polysaccharides [23].

Influence of extraction parameters

An experimental design was used to study the influence of different parameters on the water-soluble components extraction conditions. Experiments were realized with the same sample of *S. chordalis* and with the protease P1 with respect to the previous results, e.g. best extraction yield, a biochemical composition rich in organic matter, and an effective antiviral activity.

As observed in Figure 2, an increase in the release of the total water-soluble compounds number was obtained when the extraction

time was increased up to 200 min, whereas at a longer time (270 min), the extraction yield remained practically the same. Nevertheless, many studies have carried out extraction with enzymes for 12-48 hours [6]. Time plays an important role in the quantity of released compounds in the short term and in the quality of these compounds in the long term. Enzymes attack first the algal biomass to release simple compounds, and with time, they access to solubilized compounds and transform them into lower molecular weight compounds.

However, the proportion of neutral sugars after optimization decreased in the present study, while the proportion of non-detected compounds increased (Table 7). This result may be explained by the solubilization of non-quantified sugars from sulfated polysaccharides. In the optimized conditions, the time of extraction is longer and the ratio E/S more important. In the opposite, the protein level increased when optimized parameters were applied. A hypothesis consisting in the formation of agglomerates between proteins and sulfate polysaccharides may explain the lower level of sulfate groups and neutral sugar contents in the WSE after optimization with P1.

Furthermore, more soluble compounds were extracted at 50°C. Nevertheless, working at 25°C seems to be as effective as at 50°C. This observation was also made by Dumay et al. [5].

Concerning the ratio E/S, results have shown that the more enzymes are added, the more important is the extraction yield, whereas no significant difference was perceptible with a ratio of 2.5 and 7.5% (samples N°3 and 11 in Table 5).

Algal material is not the appropriate and specific substrate for enzymatic activity. In our study, the enzymes facilitate the liquefaction and the solubilization of water-soluble organic compounds. This type of RSM required fewer experiments and facilitated the arrangement and the interpretation of the effects of the parameters tested. Some unexpected results for certain extraction conditions were found, and they would be worth testing to anticipate the industrial scale for lower costs in energy and in enzymes.

Environmentally-friendly alternative

The statistical model for predicting the optimization response values was checked by using the selected optimal conditions (Table 6). The quantity of available water soluble compounds from *S. chordalis* increased significantly by 30% after the action of the enzyme P1 and non-significantly by 4% after optimization (Table 7). However, the extraction conditions for enzyme screening were already close to the optimal conditions. The optimization of extraction conditions confirmed the efficiency of the enzyme-assisted extraction by the repeatability of extraction yields. The antiviral activity is kept after the optimization, but it is significantly less important, which can be explained by the smaller proportion of available sulfated saccharides in extracts.

In conclusion, our study confirms that enzyme-assisted extraction can be used as a promising alternative and sustainable technique to recover active compounds. Compared to an aqueous extract in the same conditions, all the commercial enzyme solutions were more effective in the release of water-soluble compounds. The choices of the nature and of the concentration of enzymes are important as well as the conditions of extraction and the biochemical composition of the raw material. The response surface is a useful and powerful methodology to analyze the influence of different parameters simultaneously on the same algal material that impact directly on the quality and on the quantity of extracted active compounds. Obtained extracts have shown

antiherpetic activities with EC₅₀ between 86.0 and 163.9 µg.mL⁻¹. This activity would be enhanced by investigating the benefit that the parameters of extraction can bring to the target sulfated saccharides. This study could serve as a base for increasing the quantity of active water-soluble compounds extracted from proliferative seaweeds. Further research focusing on the scale-up of the process design and on the optimal conditions for specific active compounds extraction is highly recommended.

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