PRE-TREATMENT OF ZOSTERA MARINA AND ULVA ARMORICANA FOR PRODUCTION OF BIOETHANOL AND BIOGAS

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Centro Ricerche Trisaia, Matera

RT/2009/11/TER

ENTE PER LE NUOVE TECNOLOGIE,
L'ENERGIA E L'AMBIENTE

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Summary

The seaweed Zostera marina and the alga Ulva armoricana have been investigated to test the effect of the steam explosion treatment on their conversion in biofuels, namely ethanol and methane. Zostera has been collected on the beach of Arcachon while Ulva in Britanny (both places in France). Several analytical methods have been tested and compared to analyse the raw materials and the derivate products. The composition has been determined in terms of carbohydrates (rhamnose, glucose, xylose, arabinose, mannose, galactose, galacturonic acid, glucuronic acid, fucose) sand and other impurities, ash, extractives (chlorophills) zosterine.

Both Zostera and Ulva have been treated by steam esplosion and the best conditions were assessed to improve sugar extractability and cellulose hydrolysis by enzymes.

An experimental design was set up and the relationships were obtained to for sugar recovery and glucose production as function of the SE severity in the range 160-180°C, 60-300 s.

Zostera marina was investigated for the bioethanol production. This biomass contains over 30% of cellulose. The effect of the impregnation with oxalic acid on the quality of the produced substrate was evaluated. Tests of SHF (Separate Hydrolysis and Fermentation) and SSF (Simultaneous Saccharification and Fermentation) were carried out at bench scale and the yield of ethanol production was optimized by varying the enzyme load, the amount of yeast and the solid to liquid ratio. Ethanol can be produced with yields over 90% by SHF with 6% of enzyme and 3 g/l of yeast. The best steam explosion treatment resulted that at 180°C, 300 s, with the addition of 20 g of oxalic acid per kg of raw Zostera.

Ulva armoricana and Zostera marina were investigated for the methane production by means of anaerobic digestion. It was tested the effect of the steam explosion on the yield and the kinetics of biogas production.

The overall biogas production of steam exploded Ulva and Zostera was higher than raw material. The steam explosion treatment positively affected the kinetics of the process.

Biogas yields resulted different between Ulva and Zostera: 0.22 m^3*/kg versus 0.11* m^3*/kg; differences in the biogas composition were also detected.*

Cost analysis was carried out on the SE process applied on the Zostera and Ulva. It is based on data collected from batch (0.5kg/cycle) and continuous plant (350 kg/h, included of the fractionation section). The treatment cost has been evaluated as a function of the plant scale. Variable and fixed cost breakdown has been detailed in the case of a 1,000 and 10,000 t/y plant.

Steam explosion process costs range between 0.25 and 0.27 €/kgDM in the case of a 10,000 t/y size plant.

Key words: *bioethanol, biogas, Zostera, Ulva, steam explosion*

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Sommario

La pianta acquatica Zostera marina e l'alga Ulva armoricana sono state studiate per verificare l'effetto del trattamento di steam esplosion sulla loro conversione in biofuels, ovvero etanolo e metano. La Zostera è stata raccolta sulla spiaggia di Arcachon, mentre l'Ulva in Britannia (entrambi i posti in Francia). Diversi metodi analitici sono stati esaminati e confrontati per analizzare le materie prime ed i prodotti derivati. La composizione è stata determinata in termini di sabbia, carboidrati (ramnosio, glucosio, xilosio, arabinosio, mannosio, galattosio, acido galatturonico, acido glucuronico, fucosio) ed altre impurità, cenere, estrattivi (clorofilla), zosterina.

Sia la Zostera che l'Ulva sono state trattate mediante steam explosion e sono state valutate le condizioni migliori per l'estrazione di zuccheri e per l'idrolisi dei polisaccaridi da parte degli enzimi.

È stato effettuato un piano sperimentale, mediante il quale sono state ottenute relazioni per il recupero di zuccheri e per la produzione di glucosio in funzione della severità di SE, nell'intervallo 160-180°C, 60-300 s.

La Zostera è stata studiata per la produzione di bioetanolo. Questa biomassa contiene più del 30% di cellulosa. È stato valutato anche l'effetto dell'impregnazione con acido ossalico sulla qualità del substrato prodotto. Prove di SHF (idrolisi e fermentazione separate) e di SSF (saccarificazione e fermentazione simultanee) sono state compiute su scala banco e le rese di produzione di etanolo sono state ottimizzate variando il carico degli enzimi, la quantità di lievito ed il rapporto solido liquido. L'etanolo può essere prodotto con rese maggiori del 90% da SHF, con 6% di enzima e 3 g/l di lievito. Il trattamento di steam explosion migliore è risultato quello a 180°C, 300 s, con l'aggiunta di 20 g di acido ossalico per chilogrammo di Zostera grezza.

L'Ulva e la Zostera sono state studiate per la produzione di metano mediante digestione anaerobica. È stato esaminato l'effetto della steam explosion sulle rese e sulla cinetica di produzione di biogas.

La produzione generale di biogas sia da Ulva che da Zostera trattate è stata superiore rispetto al materiale grezzo. Il trattamento di steam explosion ha quindi influenzato positivamente la resa e la cinetica del processo.

Le rese di Biogas sono risultate differenti fra Ulva e Zostera: 0,22 m^3/kg contro 0,11 m^3/kg; sono state inoltre rilevate differenze nella composizione del biogas.

È stata effettuata l'analisi dei costi sul processo di SE applicato su Zostera e su Ulva. Essa è stata basata sui dati raccolti da prove condotte su impianto batch (0.5kg/ciclo) e su impianto continuo (350 kg/h, incluso della sezione di frazionamento). Il costo di trattamento è stato valutato in funzione della scala dell'impianto. La ripartizione dei costi variabili e fissi è stata dettagliata nel caso di un impianto di 1.000 e 10.000 t/anno.

I costi del trattamento di steam explosion variano fra 0,25 e 0,27 €/kg_{DM} nel caso di un impianto con taglia di 10.000 t/anno.

Parole chiave: bioetanolo, biogas, Zostera, Ulva, steam explosion

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Introduction

The present report describes the results of the research activities carried out at ENEA during the triennial BIOPAL project. The project was co-participated with several European partners and co-financed by the V framework of the EU. Its main goal was to valorise the huge amount of beached algae collectable as residue on the north European coasts. The ENEA activities was addressed to valorise the algae as source of biofuels also by means a pre-treatment with the steam explosion (SE) process. The ENEA's research activity was divided in five tasks:

I. Pretreatment by steam water and raw material characterization;
II. Improved pretreatment and extractions;
III. Characterization of products and by-products from steam explosion;
IV. By-products exploitation: bioethanol and biogas from Zostera and Ulva;
V. Techno-economic analysis of algae pre-treatment.

In the section I, the effects of the steam explosion process, a hydrothermal treatment employed to fractionate the lignocellulosic biomass, were investigated on the new material: Zostera and Ulva. The effects were studied in terms of the material dissolution by aqueous extraction. At the same time Zostera and Ulva were examined to identify their constituents by means the analytical methods employed in the ENEA laboratory to characterize the biomass; literature references were also taken in consideration.

In the section II, the SE treatment was optimized by means of an experimental design to assess the best conditions for the sugars recovery by aqueous extraction and/or enzymatic hydrolysis; the relationships between the responses and SE parameters have been obtained.

In the section III, the analyses of the sugars were carried out on the streams of the SE process to verify the modification of the biomass composition as function of the treatment temperature and time.

In the section IV, the bio-processes of the enzymatic hydrolysis and fermentation to produce bio-ethanol and of anaerobic digestion to produce biogas were assessed by optimizing the involving parameters and evaluating the steam explosion effects on the process yields.

In the section V, the economic analysis was carried out to determine the costs of the steam explosion treatment applied on the Zostera and Ulva: the cost analysis was dimensioned to the algae availability.

I: Pretreatment by Steam Water and Raw Material Characterization.

1. THERMAL TREATMENT OF RAW MATERIAL

1.1 Steam Explosion Pre-treatment and Plant Description

Zostera marina and *Ulva armoricana* have been steam exploded using a STAKETECH batch reactor which is a pressure vessel made of stainless steel having a capacity of 10 liters. The reactor chamber is surrounded by a steam jacket to ensure a stable temperature of treatment and to minimize the steam condensation (Figure 1).

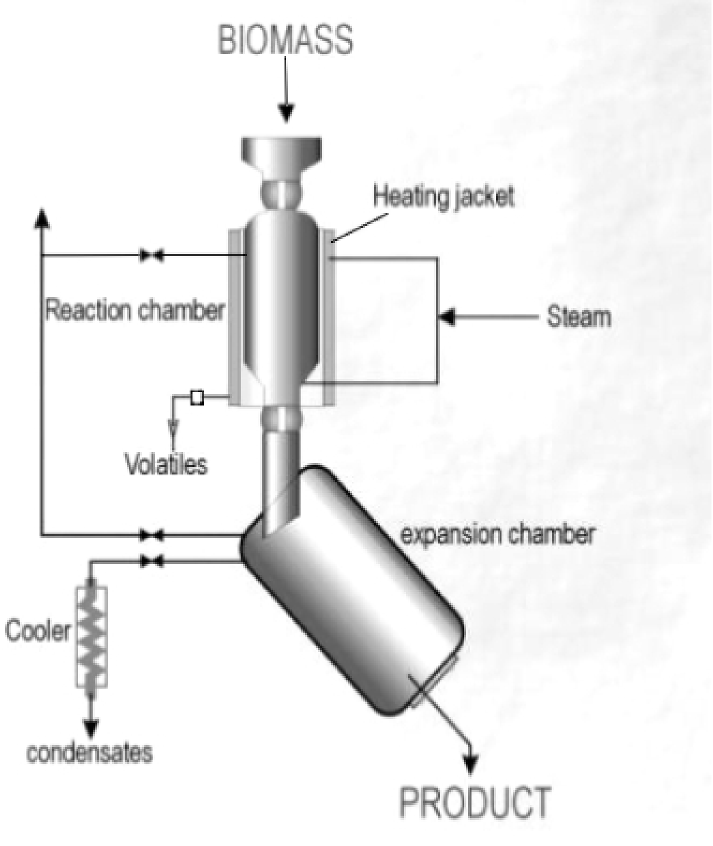


Figure 1 - Scheme of SE batch reactor.

100 g of dried sample have been humidified at 50% and manually loaded in the reactor through a pneumatic loading valve, then the pneumatic valve has been closed and the steam injected until the treatment time elapsed. At the end of the treatment, a blow valve has been opened and pressure dropped suddenly (explosion) in a very short time. The product has been recovered in a 10 liters tank (Figure 2).

A condenser is connected with the storage tank in order to recover and remove as a liquid waste the volatile organic compounds that are produced during the process.

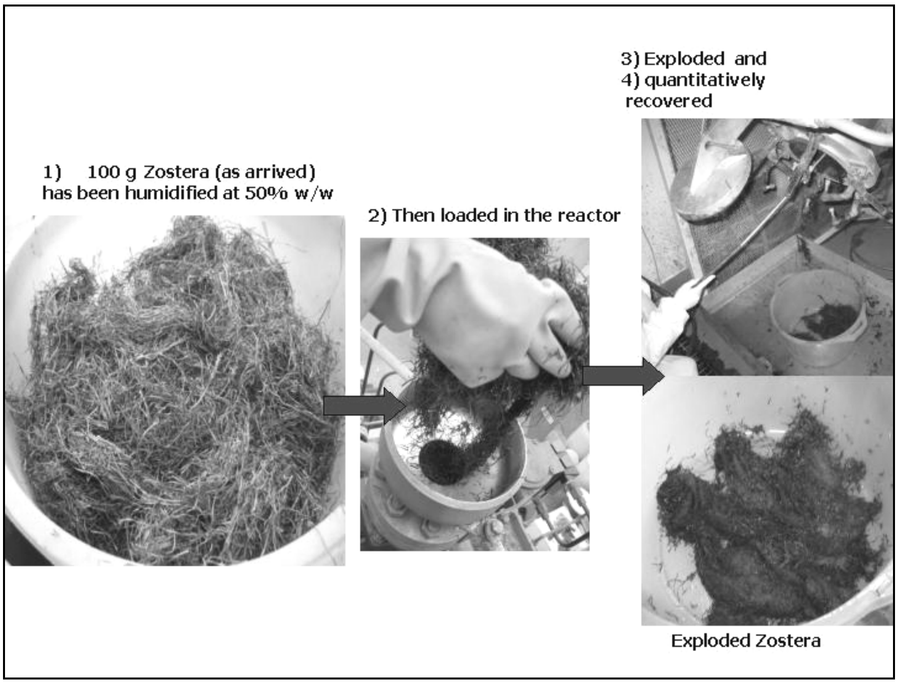


Figure 2 – Procedure of SE treatment.

The SE severity (Ro) can be related at time and temperature by the following relation:

$$Ro = t \cdot \exp\left(\frac{T-100}{14.75}\right)$$

(Where T is the saturated steam temperature in °C and t is the reaction time in minutes).

1.2 Choosing the SE severity parameter

References about steam treatment (SE) of algae and marine plant are rare. In the case of *Gracilaria dura* SE was employed to enhance agar recovery [1]. The authors tested a range of temperature and retention time and the product obtained is observed by scanning electron microscopy (Table 1).

Table 1 – Scanning electron microscopy observation on the thallus structure after steam explosion of Na_2CO_3 pre-conditioned algae [1].

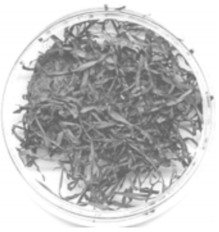
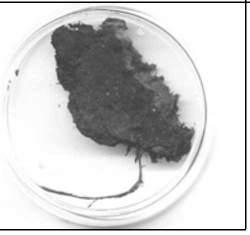
Temperature (°C)	Pressure (MPa)	Time (s)	Overall structure
190	1.1	120	Completely Destroyed
190	1.1	20	Completely Destroyed
180	0.9	60	Completely Destroyed
180	0.9	45	Completely Destroyed
180	0.9	30	Completely Destroyed
150	0.3	60	Partly Destroyed
150	0.3	30	Partly Destroyed
150	0.3	15	Apparently intact
140	0.2	100	Partly Destroyed
140	0.2	60	Partly Destroyed

Completely Destroyed: original thallus structure only partly recognizable.
Partly Destroyed: no original thallus structure recognizable.

1.3 *Steam exploded products*

Analogously to the results obtained with the Gracilaria, we have performed two treatments: at 150 °C to preserve the biomass structure, and at 190 °C to have more structural modification. In Table 2 are reported the investigated parameters.

Table 2 - Conditions of SE treatment investigated on Zostera and physical aspect of the products.

Log Ro: 1.2 Temperature: 150 °C Time: 30 s 	Log Ro: 1.9 Temperature: 150 °C Time: 165s 
Log Ro: 2.2 Temperature: 150 °C Time: 300 s 	Log Ro: 2.9 Temperature: 190 °C Time: 120 s 
Log Ro: 3.3 Temperature: 190 °C Time: 300 s 	

The picture magnification (50x) obtained by optical microscope shows the differences of aspect between raw material and steam exploded product (Figure 3). Leaves of exploded product are amassed because of increased surface and cohesion due to SE treatment.

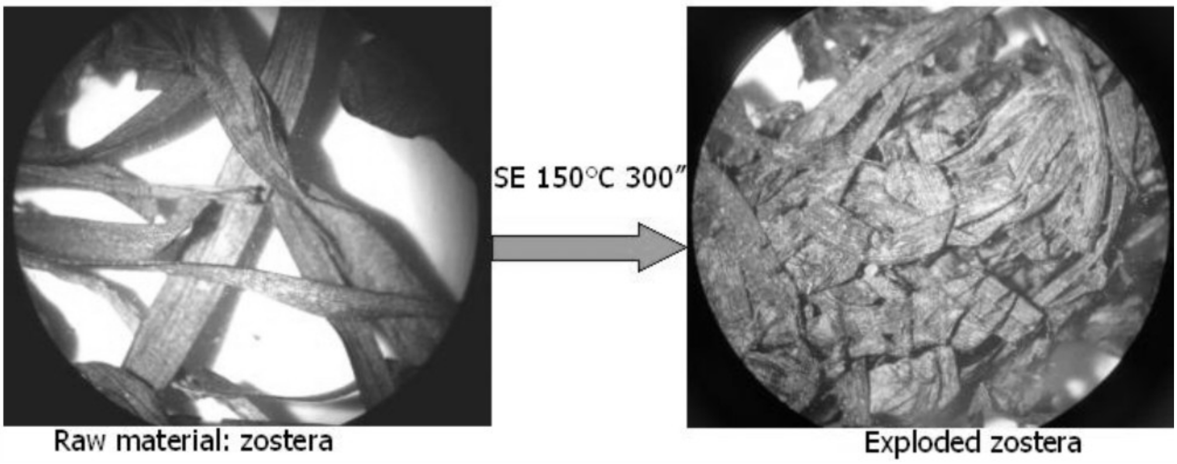


Figure 3 – Morphology of the raw material and SE product.

1.4 Water washing and fraction recovery

The product is reach of liquor that can be separated by extraction. The washing is carried out at 65 °C to extract soluble pectin as co-products. The suspension is then squeezed with a hand-press (Figure 4) to separate the solid and liquid phases.

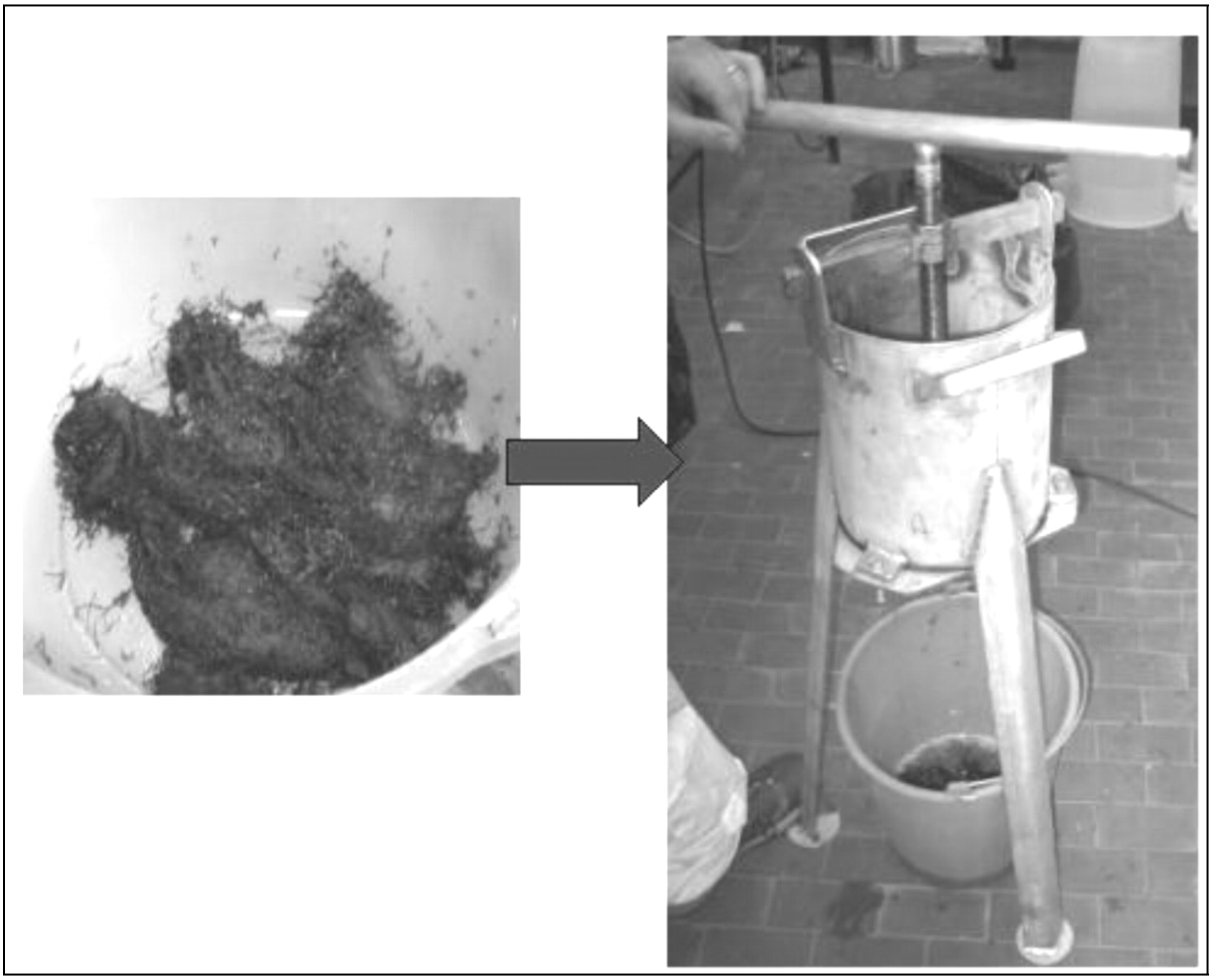


Figure 4 – Squeezing of SE biomass with filter-press.

After the separation, the solid product was dried at 40 °C and stored in plastic bag. The aqueous extract was frozen and stored for the chemical analyses.
Dry matter of extracted liquor is determined to quantify the amount of soluble fraction, so the ratio of extracted material and the insoluble residue was determined (Figure 5).

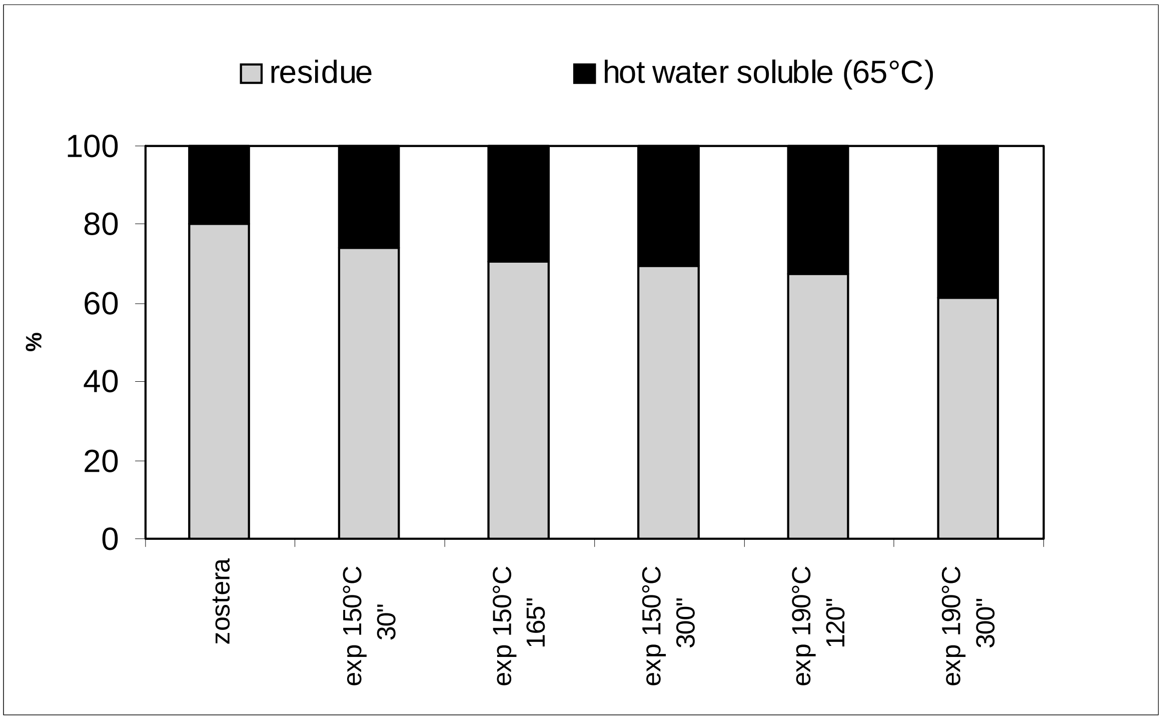


Figure 5 – Recovery of water soluble material and solid residue after water washing in Zostera before and after Steam Explosion.

As shown in Figure 5, high severities of SE treatment increase the recovery of substances in the aqueous fraction. This is expected, because SE cleaves the chemical bonds between the macromolecules of the biomass enhancing its fractionation. Pectin is soluble in hot water and its main constituent is the galacturonic acid [2].

The Zostera and Ulva were also extracted with alkaline solution (1.5% NaOH) at 90 °C. It was evident that the solubility of the materials increased with the steam explosion severity. The Zostera as raw material is soluble in warm water up to 20% while at the highest severity of 190 °C, 5 min the solubility is almost doubled (38%). The same is pointed out for Ulva that, as raw material, is soluble up to 20%, while at the highest steam explosion severity (170 °C, 3 min) a solubility of 43% can be attained.

Solubility in alkali does not increase in raw material respect to the simple water extraction. Steam exploded materials have a slight solubility in alkali (respect to the simple water solubility) and this increases with severity. In the case of Zostera up to 4% of the DM is soluble, while for Ulva 0.8 % solubility has been detected only at 170 °C for 3 min (Figures 6 and 7).

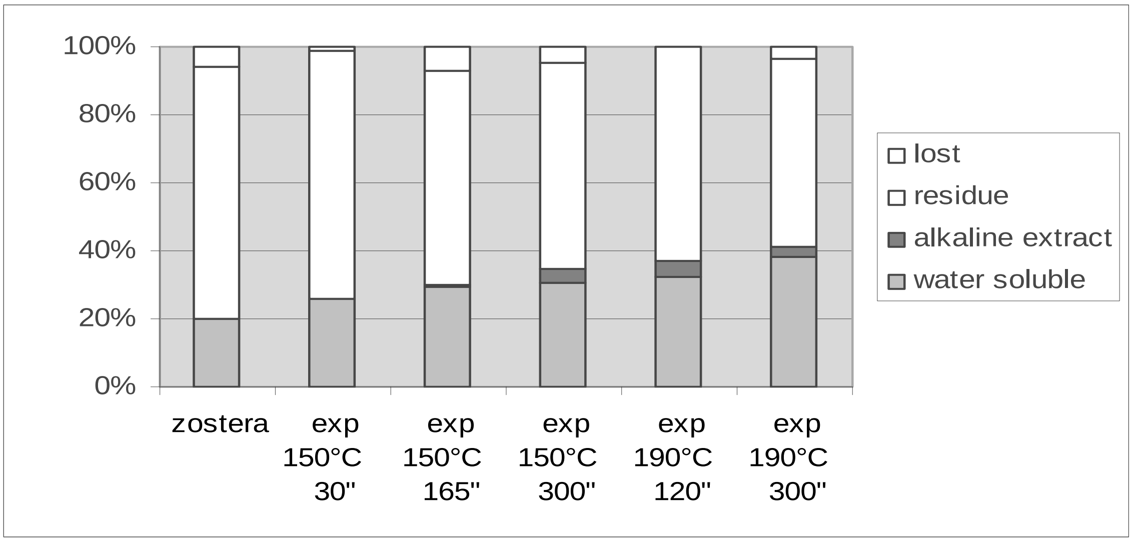


Figure 6 – Recovery of water soluble material and solid residue after water and alkaline washing in *Zostera* before and after Steam Explosion.

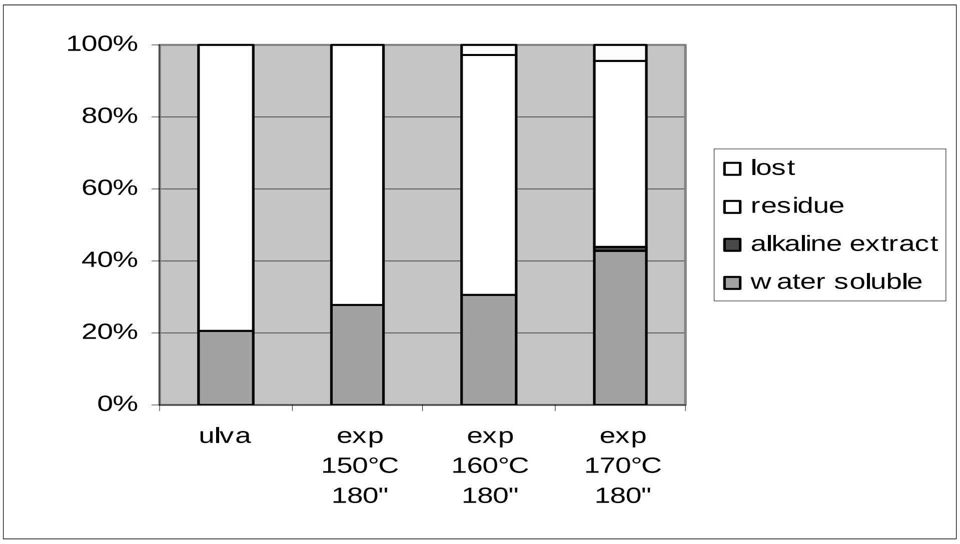


Figure 7 – Recovery of water soluble material and solid residue after water washing and alkaline washing in Ulva before and after Steam Explosion.

2. RAW MATERIAL CHEMICAL CARACTERIZATION

2.1 General information of examined materials

In Table 3 is reported the botanical classification of the used biomasses.

Zostera is a grass like flowering plant with dark green, long, narrow, ribbon shaped leaves 20-50 cm in length (exceptionally up to 2 m long) with rounded tips, 4-10 mm wide, with 5-11 veins and rounded leaf tips, sometimes with a sharp point (mucronate). Leaf sheath forms a tube around stem. Reproductive shoot, terminal, branched and up to 15 m long. The seeds are ovoid or ellipsoid with 16-25 distinct ribs. Rhizome with fiber bundles in the outermost layer of cortex. Leaves shoot from a creeping rhizome that binds the sediment. Leaves and rhizomes contain air spaces, lacunae, that aid buoyancy. Numerous flowers occur on a reproductive shoot similar to those of terrestrial grasses. Zostera forms dense swards in the subtidal, supports a diverse fauna and flora and may act as a nursery for fish and shellfish. Dense swards are found primarily on sand to fine gravel in the subtidal, typically down to 4 m, in sheltered waters such as shallow inlets, bays, estuaries and saline lagoons. The material that has been used in this study has been collected in Arcachon, a french beach on the Atlantic Ocean

Ulva has a laminar form; its presence is typical in eutrophycs zone. Ulva is an alga naturally present on our littoral, it is in 2 forms:

- Benthonic: fixed at the underwater funds.
- Pelagic: in suspension in water, moving with the liking of the currents. It is this form which is responsible for the green tides.

Blades of *Ulva armoricana* are variable in shape, fragile, easily torn, and 30-55 µm thick in middle and apical regions; they have pale green color, crumpled texture that creates an irregular light-reflecting surface and the presence of tooth-like protuberances at the margin of the blade. *Ulva armoricana* has longitudinal ribs containing closely packed bundles of rhizoids, except in the youngest thalli. *Ulva armoricana* has the polygonal (never rounded) cells in the mid-thallus region, and quadrangular cells arranged in small longitudinal rows in rapidly growing parts.

The material that has been used in this study has been collected in Britanny (France) on June 2003 and dried (not washed).

Table 3 – Taxonomy of studied species.

	Zostera marina	*Ulva armoricana*
Phylum	Anthophyta (Flowering plants)	Chlorophyta
Class	Liliopsida (Monocotyledons)	Chlorophyceae
Order	Potamogetonales	Ulvales
Family	Zosteraceae	Ulvaceae
Genus	*Zostera*	*Ulva*
Species	*marina*	*armoricana*

2.2 *Chemical composition of algae in literature references*

The Table 4 reports the chemical composition of some representative seaweed. All figures (except for water), are given as grams per 100 grams of dry matter.
In regard to the amount of protein, the convention is to convert the total nitrogen into protein by multiplying the former by 6.25.
These figures were taken, with slight modification, from Indergaard & Minsaas (1991).

Table 4 - Chemical composition of seaweeds: references.

	Ascophyllum nodosum	*Laminaria digitata*	*Alaria esculenta*	*Palmaria palmata*	*Porphyra sp.*	*Porphyra yezoensis*	*Ulva species*
Type	Brown	Brown	Brown	Red	Red	Red	Green
Water (%)	70-85	73-90	73-86	79-88	86	nd	78
Ash	15-25	73-90	73-86	15-30	8-16	7.8	13-22
carbohydrates	-	-	-	-	40	44.4	42-46
Alginic acid	15-30	20-45	21-42	0	0	0	0
Xylans	0	0	0	29-45	0	0	0
Laminaran	0-10	0-18	0-34	0	0	0	0
Mannitol	5-10	4-16	4-13	0	0	0	0
Fucoidan	4-10	2-4	nd	0	0	0	0
Floridoside	0	0	0	2-20	nd	nd	0
Protein	5-10	8-15	9-18	8-25	33-47	43.6	15-25
Fat	2-7	1-2	1-2	0.3-0.8	0.7	2.1	0.6-0.7
Tannins	2-10	c. 1	0.5-6.0	nd	nd	nd	nd
K	2-3	1.3-3.8	nd	7-9	3.3	2.4	0.7
Na	3-4	0.9-2.2	nd	2.0-2.5	nd	0.6	3.3
Mg	0.5-0.9	0.5-0.8	nd	0.4-0.5	2.0	nd	nd
Iodine	0.01-0.1	0.3-1.1	0.05	0.01-0.1	0.0005	nd	nd
References	[3]	[4, 5, 6, 7]	[4, 5, 6, 7]	[8]	[9, 10]	[10]	[9, 10]

Table 5 reports the methods and compositions found in literature.

Table 5 – Chemical composition of marine plants and algae: references.

Biomass	Element	Content	Method	References
Zostera marina	Non-structural carbohydrate: Soluble sugars (sucrose, glucose and fructose); Starch	9.8% (in the leaves) 25% (in the rhizomes) 17.4% (in the roots)	80% ethanol 30% perchloric acid	[11]
Zostera marina	Carbohydrate: sugars (sucrose, glucose fructose); Starch	350 mol/gFW (leaves) 10-20 mol/gFW	Hot Ethanol 90% at 80 °C, 15 min 1 N KOH room T, Overnight.	[12]
Zostera marina Higher plants, Green algae	Chlorophylls a + b	2 mg/g FW	Extraction with ice cold acetone (90% v/v)	[13]
Zosteraceae	Pectic polysaccharide	10-12%	Methanol extraction, separation with ammonium oxalate 1%	[2]
Marine macroalgae	Carbohydrate	19-25%	Acetone (10 ml/g); Hydr. 72% H2SO4 , 1 h, 30 °C	[14]
Ulva rigida	Neutral sugars (Ulvan fraction)	Sulfate 35.9 (mol %) Uronic acid 24.9 (mol %) Rhamnose 23.3 (mol %) Xylose 10.9 (mol %) Glucose 3 (mol %)	Extraction with ammonium oxalate, Hydrolysis with 2 M H_2SO_4 for 2 h at 100 °C	[15, 16]
Ulva armoricana	Ulvan	Rhamnose 51.1 (mol %) Xylose 8.3 (mol %) Glucose 2.5 (mol %) Galactose 2.8 (mol %) GluA 19.9 (mol %) IdoA (mol%)	Extraction and purification	[17, 18]
Brown algae	carbohydrates	65.8%	Phenol-sulfuric acid	[19]
Brown algae	Fucoidan	3.8%	HCl 0.1 M Methylation analysis Extraction with Triton X-100, acid and alkali	[20, 21, 22]
Brown algae	Laminaran, Fucoidan Crude alginate Insoluble fraction	1-22% DM 10-67% DM 3-59% DM	N HCl treatment, Et2O precipitation; 1%Na2CO3 treatment, EtOH precipitation; Insoluble fraction in Na2CO3	[23]
Phaeophycea	Acidic polysaccharides (alginic acid, ylofucan and fucans)		NaCl 0.1 M extraction and purification A. C. Hydrolysis 1 or 4 N HCl 100 °C, 2 h	[24]
Lessonia trabeculata	Uronic acid Glucose	Total hydrolysis of alginic acid	80% H_2SO_4 at 0 °C; 90 % formic acid at 100 °C HCl 3 M	[25]
Plants, animals, algae bacteria and fungi	Uronic acid Glucose		72% H_2SO_4 1 h, 30°C; 3% H2SO4 3 h, 100°C; 2M TFA 1h, 121 °C Methanolysis+TFA	[26]

2.3 Methods of raw material Characterization

2.3.1 Raw material pretreatment

Raw material was separated from sand and salt by cold water washing. Then samples were ground at 50 Mesh and dried at 40°C overnight.

2.3.2 The extractives

The extractive content has been determined by soxhlet extraction using a mixture of toluene and ethanol 2:1 for 6 hours (CPA G-13 method).

2.3.3 The pigment extraction

The treatment with cold acetone and cold methanol (10 ml/g $_{\text{dried sample}}$) was used as selective method to extract fat, non polar compounds and chlorophyll pigments.

Extracted materials were analyzed by UV-VIS spectrophotometer instrument (HITACHI Co. V2000) operating in scanning modality (400-720 nm).

2.3.4 Determination of Chlorophylls a, b

Chlorophyll is determined following the method reported by Jeffrey S.W. and Humphrey G.F. (1975) based on revised extinction coefficients of chlorophylls a and b and utilizing the equations further reported.

The equations may be employed to assay a variety of mixtures of chlorophylls which are typically found in higher plants, green and brown algae, diatoms, chrisomonads, dinoflagellates and cryptomonads.

Pigment is extracted from about 3 gram of sample with methanol.

The solvent is removed by evaporation, and then the residue is dried in the oven at 60 °C.

The dried pigment is solubilized in acetone/diethyl ether 9:1 and analyzed by UV-VIS spectrophotometer (Hitachi Co v2000).

Results are expressed in µg/ml using 1 cm cell path-length.

The symbol E_x denotes the extinction at wavelength x. For 72 mg of pigment in 50 ml of solution (1.44 mg/ml) we measured E_{664} and E_{647}.

For the green algae the relations reported are:

$$Chlorophills\ a = 11.93E_{664} - 1.93E_{647}$$
$$Chlorophylls\ b = 20.36E_{647} - 5.50E_{664}$$

2.3.5 Carbohydrate separation and determination

Severall tests were performed to isolate and determine the carbohydrates content:

1. Klason lignin treatment. About one gram of extracted material was used. The first hydrolysis was carried out using 15 ml of H_2SO_4 (72% w/w) under manual mixing, then the samples were put in a water bath at 20 °C and carefully mixed at times for 2 hours. The secondary hydrolysis has been carried out by adding 560 ml of water in order to reduce the H_2SO_4 concentration down to 3%. The solution was kept at its boiling point and constant volume for 4 hours. Filtration was carried out to separate the acid insoluble residue **(AIR)** using vacuum pump and glass filter. The insoluble residue is dried, weighted and analyzed by FTIR.

2. Ethanol precipitation method. One gram of dried grounded sample was first extracted with cold methanol (10ml) to extract the pigments and then treated to obtain 3 fractions:
   - A first fraction **(F1)** was obtained from the treatment with 10ml of HCl 0.1N (2h) followed by a precipitation with ethanol (40ml);
   - A second fraction **(F2)** was obtained from the HCl insoluble residue treated with 1% Na_2CO_3 (50ml) and then precipitate using 200ml of ethanol;
   - A third fraction **(F3)** represents the insoluble Na_2CO_3 fraction.

   All the fractions were hydrolyzed following the Klason procedure as reported above. In the Figure 8 is shown the scheme of the employed analytical procedure.

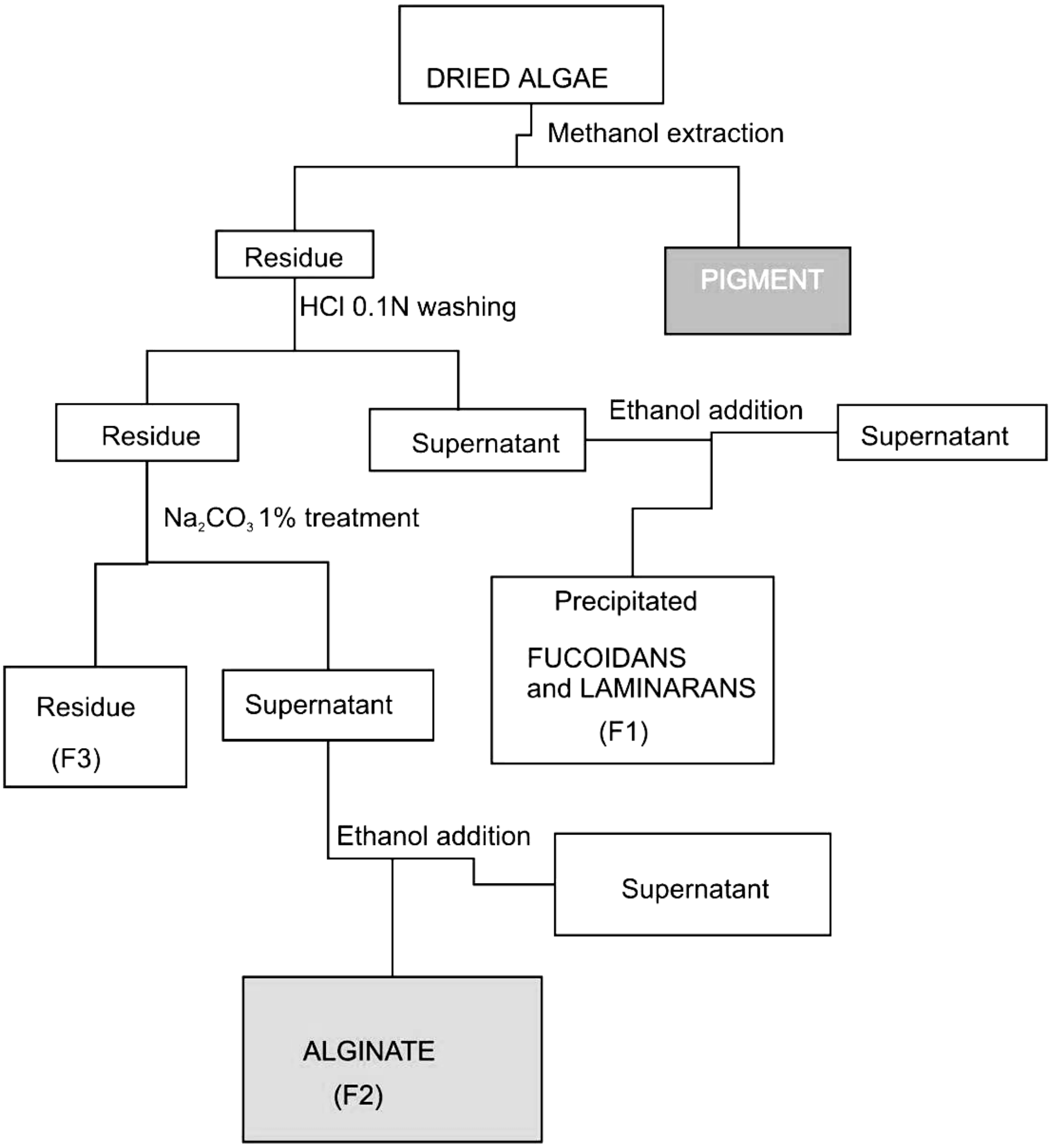


Figure 8 – Scheme of analytical procedure.

3. *Combined extraction and hydrolysis* of hot water extractable material by soxhlet apparatus. About 1 gram of raw material has been weighted in glass fiber thimble and submitted simultaneously to:
    - Zosterin extraction by water content in a flask;
    - Hydrolysis of extracted zosterin by 200 ml H_2SO_4 1 N.

   The same method has been applied to material without chlorophyll.

4. *Extraction of zosterine with ammonium oxalate*. After extraction with methanol to remove the pigment, the residue was treated with formaldehyde 1% (10 ml for two times) for 16h at 20 °C. The solid residue was treated with dilute HCl (pH 4) for 4h at 50 °C, and then separated and washed with cold water. Zosterine was extracted three times with 1% aqueous ammonium oxalate for 3-5 h at 70 °C and precipitated by adding the extract to 4 volumes of methanol. Zosterine was reprecipitated from 80% ethanol and dried at 40 °C over night. Zosterine (1 g) was hydrolyzed with 2N H_2SO_4 (100 ml) for 16h at 75 °C).

The analysis was carried out by HPIC (DIONEX DX500) with Carbopac PA1 column, NaOH 2.4 mM as eluent (flow rate 1.0 ml/min., at 30 ^{o}C) and ED40 detector with gold working electrode.

Soluble lignin was determined by UV spectrophotometer (HITACHI Co. V2000) at 205 nm, assuming an extinction coefficient of 110 $dm^3/g \cdot cm$ (TAPPI Useful Methods 250).

2.3.6 The ash

The ash content was determined by combustion at 600 ^{o}C (ASTM-1102, modified) and reported afterwards as “inorganic”.

All the determinations were carried out in duplicate.

2.4 Results of analytical determination

The composition of Zostera and Ulva are reported in Table 6.

Table 6 – Composition of Zostera and Ulva (w/w_{DM}, %).

	Zostera	**Ulva**
Sand and other impurities	2-3	19.4
Ash	13.7	21.3
Extractives	6.7	
Pigment (methanol extraction)	1.5	1.73
Pigment (acetone extraction)	2.2	2.9
Zosterine	7.0	
F1[a]	0.5	6.0
F2[b]	-	10.2
F3[c]	-	45.4
Total carbohydrates (aldoses)	22.7	
Rhamnose		
Glucose	16.4	8.5
Xylose	2.5	1.3
Arabinose		4.5
Mannose	-	
Galactose	2.2	0.2
Galacturonic acid	1.0	nd
Glucuronic acid	nd	
Fucose		
Chlorophyll a		267 µg/g
Chlorophyll b		288 µg/g
AIR[d]	27.0	15

a: fucoidans; b: crude alginate; c: insoluble residue, [23]; d: Acid Insoluble Residue (Klason procedure).

2.4.1 Visible spectra

Figure 9 shows the visible spectra of the organic solvent extraction performed on the crude zostera. Methanol result more selective then acetone in chlorophyll extraction because in the spectrum are not present the two signal (530 and 610 nm) that increment the percentage of material extracted.

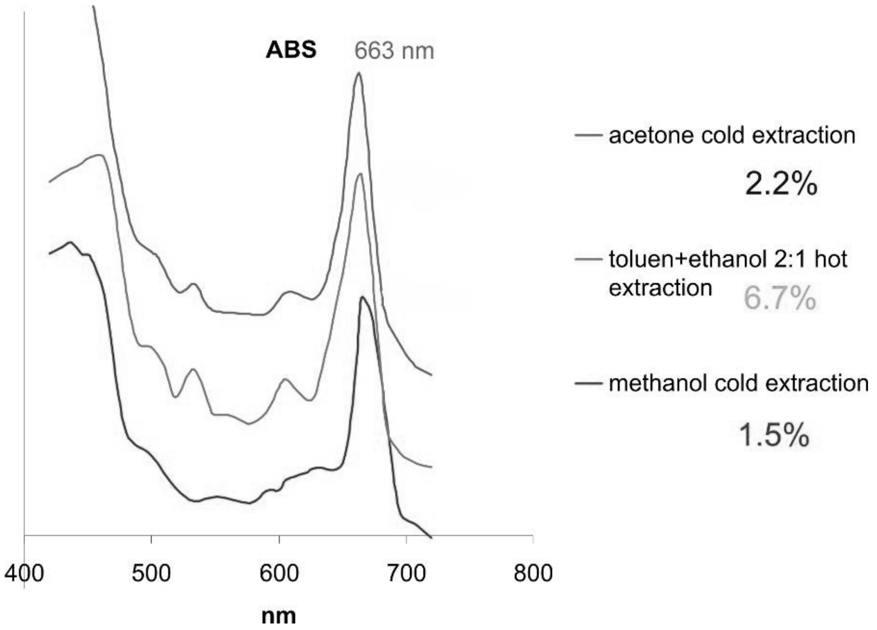
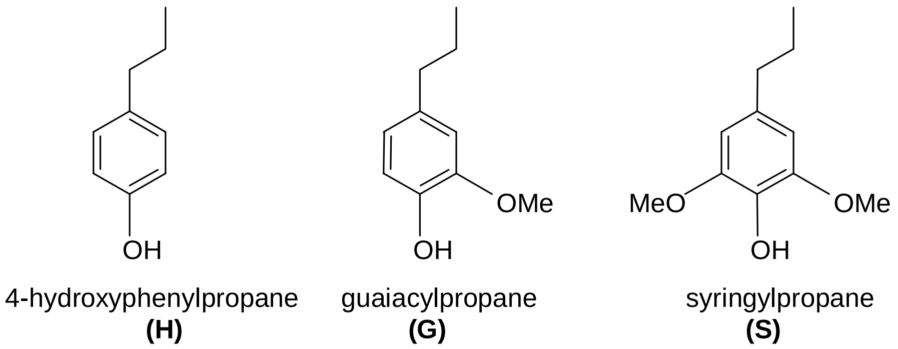


Figure 9 – Visible spectra of extracted materials on crude Zostera.

2.4.2 <u>FTIR of Klason AIR from Zostera and Ulva</u>

The acid insoluble residue obtained from the Klason hydrolysis procedure of **lignocellulosic materials** is a polymer made by the basic units: 4-hydroxyphenylpropane (H), guaiacylpropane (G) and syringylpropane (S):



It is called with the general name of *lignin* and has a chemical structure that is specific of each lignocellulosic matrix. Figure 10 shows a possible structure of a lignin from softwood:

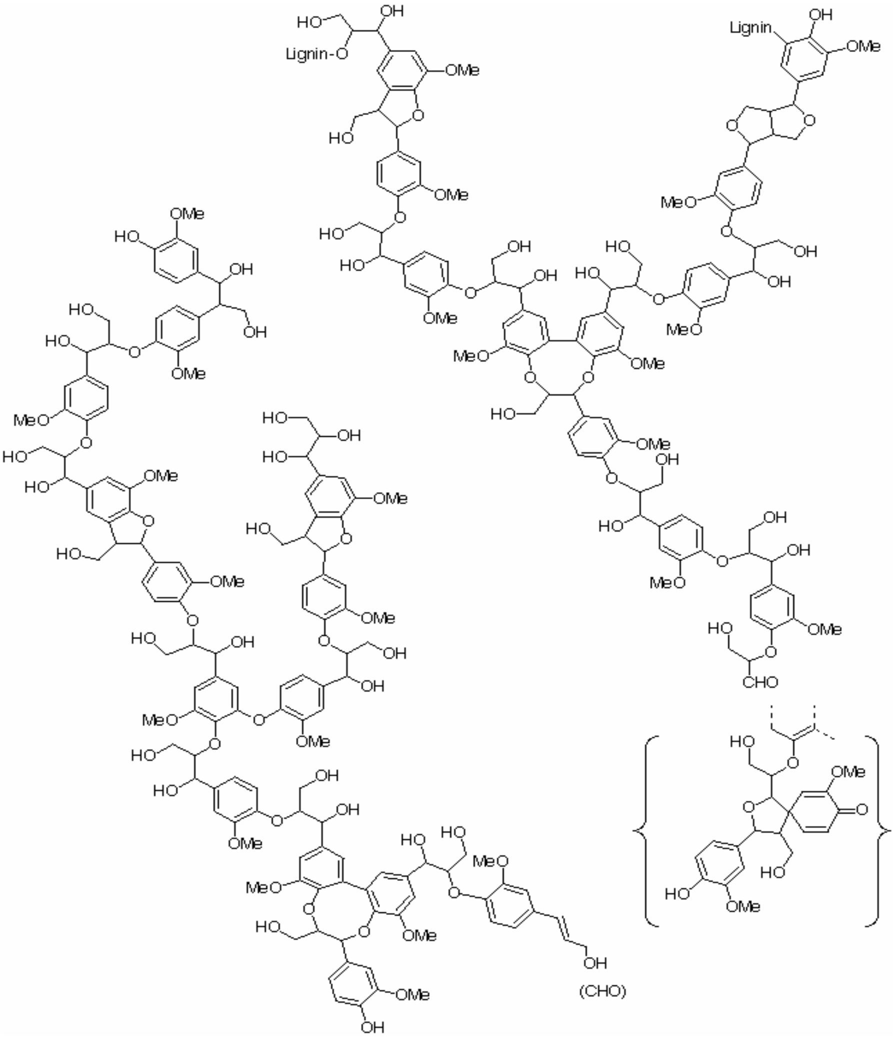


Figure 10 – Model structure of softwood lignin.

Depending on the relative abundance of the basic units and the degrading products obtained by means of some oxidative reactions, it is possible to classify lignin into three groups:

- G type: lignin giving rise mainly to vanillin
- GS type: lignin giving rise to vanillin and syringaldehyde
- HGS type: lignin giving rise to 4-hydroxy-benzaldehyde, vanillin and syringaldehyde

Via IR spectroscopy lignins from different of the above classes present qualitative futures that make possible to distinguish a type from another.

In Figure 8 is shown a typical set of IR spectra of G and GS type lignin relevant to the range 2000-800 cm $^{-1}$, the fingerprint region of these materials.
In this spectral region the bands are mainly due to vibration from carbonyl and carboxyl groups, aromatic rings, alcohol and ethers groups and coupling of them.

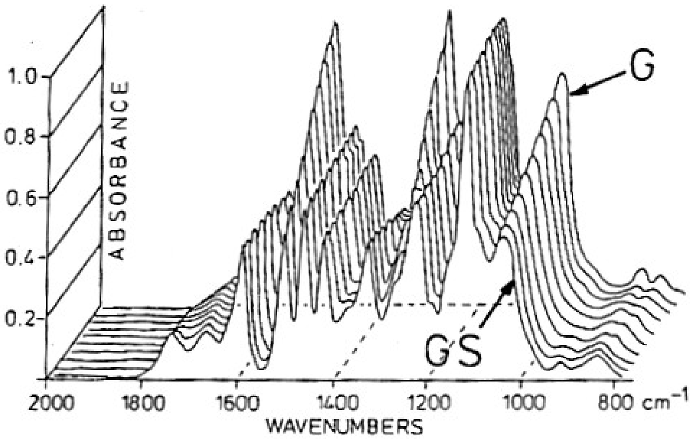


Figure 11 - Spectral continuum of GS lignins. (Ref.: O.Faix. Classification of lignin from different botanical origins by FTIR spectroscopy. *Holzforschung, 45 (1991) Suppl., 21-27*).

Lignin of the HGS type is characterised from an IR spectra very similar to the GS lignin, however the higher intensity of the band present at about 835 cm^{-1} makes possible to distinguish the former from the latter.
Further typical bands of lignin are in the ranges 3500-3300 cm^{-1} (O-H stretching from phenol groups) and 2950-2850 cm^{-1} (aliphatic C-H stretching) but since they do not show any pronounced differences relevant to the classification, in the Figure 11 the region at wavenumbers higher then 2000 cm^{-1} is not reported.

2.4.3 IR spectra of Zostera and Ulva

The profiles shown in the Figure 10 in its whole is quite specific of lignin and in this report has been adopted as a reference to evaluate the nature of the Klason residues obtained from the samples of SE Zostera and Ulva.

Sample Preparation

The residue from the Klason hydrolysis of steam exploded samples of both Zostera and Ulva were submitted at the IR spectrum acquisition in the region 4000 ÷ 650 cm^{-1}.
The spectra were collected on a Perkin Elmer 2000 FTIR spectrometer using the KBr disc method. The spectral resolution was 2 cm^{-1}.
Each spectrum was the average of 16 acquisitions, no elaboration were performed on it.
The two spectra are shown in Figure 12 and 13 respectively:

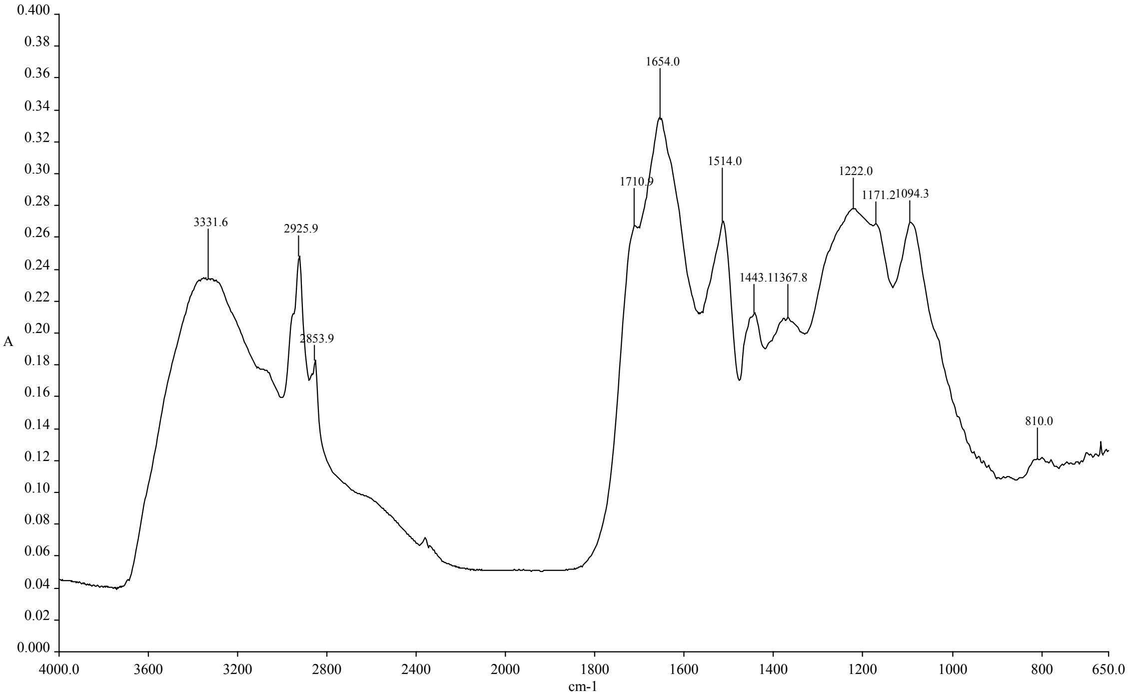


Figure 12 - FTIR spectrum of the acid insoluble residue from Steam Exploded Zostera.

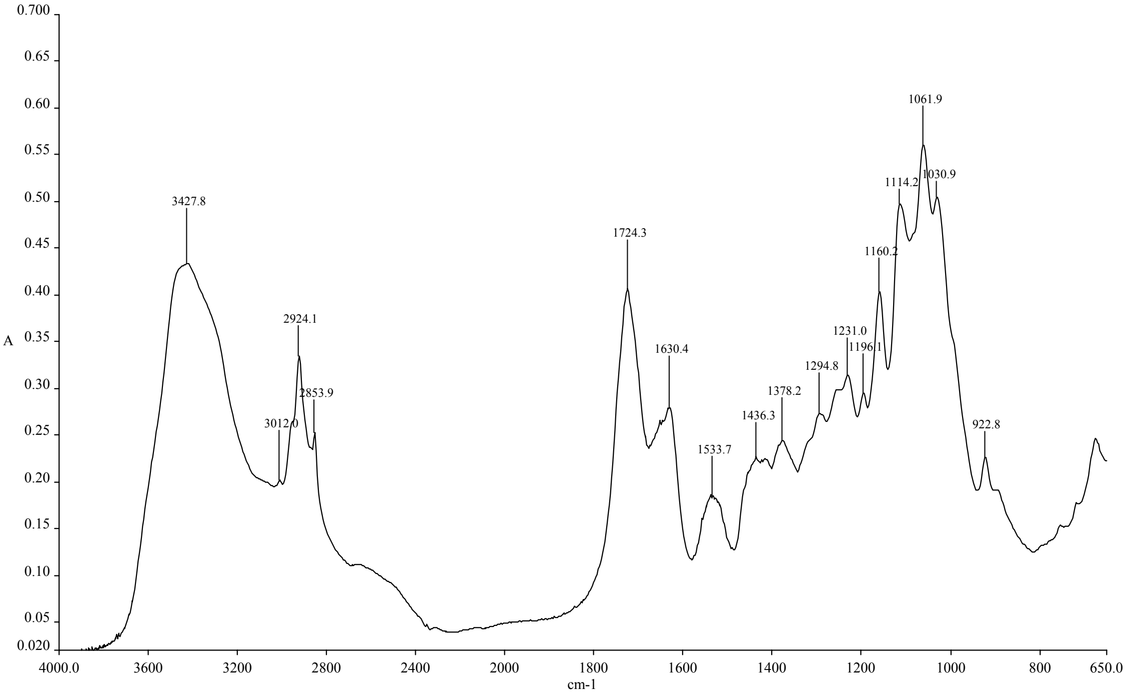


Figure 13 - FTIR spectrum of the acid insoluble residue from Steam Exploded Ulva.

Comparing the fingerprint region (1800-650 cm^{-1}) of the Klason residues with respect to the typical spectra of lignins, it appears that **there is no relevant matching among the three spectra**. It can be inferred that the acid insoluble residue is not “simple” lignin as we intend it from the terrestrial lignocellulosic biomass, because the material lack of the typical functional groups guaiacil and syringil units.

For an unknown molecular structure the exact peak assignment to a well defined functional group is quite difficult to be carried out on the unique basis of their IR spectra, moreover we have tentatively done such work on the basis of general IR absorbance Tables and similarities with the spectra of carbohydrates such as cellulose, hemicellulose and alginate. Further work should be needed to clarify the chemical structure of the acid solid residue.

In Table 7 are listed the wavenumbers of the main spectral bands and a possible assignment for some of them.

Table 7 - Assignment of the main bands for Klason residue from steam exploded samples of Zostera and Ulva (N.A. no assignment).

Zostera	
ν (cm^{-1})	Band Origin
3332	Stretc. OH
2926	Asym Stretc. CH_2
2854	Sym Stretc CH_2
1711	Stretc. C=O in unconjugated ketone;
1654	Stretc. C=O in conjugated ketone;
1514	Aromatic skeletal vibration
1443	Aromatic skeletal vibration
1368	N.A
1222	N.A
1171	Stretc C-O-C (H).
1094	Stretc. C-O-C (H)
810	N.A.
Ulva	
ν (cm^{-1})	Band Origin
3428	Stretc. OH
2925	Asym Stretc. CH_2
2854	Sym Stretc CH_2
1724	1) Stretc. C=O in unconjugated carbonyl; 2) Stretc. C=O in carboxylic acid involved in H-bonded network
1630	Stretc. C=O in conjugated carbonyl
1534	Aromatic skeletal vibration
1436	Aromatic skeletal vibration
1378	N.A.
1295	N.A.
1231	N.A.
1196	N.A.
1160	Stretc. C-O-C (H)
1114	in carbohydrate-like network
1062	
1031	
923	Bending out of plain arom. C-H

II: Improved Pretreatment and Extractions

1. INTRODUCTION

After the characterization of the raw materials (*Zostera marina* and *Ulva armoricana*) and the preliminary investigation on the steam explosion treatment on algae, the activity was addressed to optimize the steam explosion treatment and extractions.

In the past, Murano *et al.* [1] optimized the steam explosion process applied on Gracilaria to enhance agar extraction. Analogously, the aim of this work was been to investigate the best parameters of the steam explosion process to enhance carbohydrates extraction from Zostera.

The innovative aspect of the treatment optimization has been the preconditioning of algae with oxalic acid solutions; indeed it is reported that the main carbohydrate, zosterine, can be extracted with ammonium oxalate solution [2].

In our case, oxalic acid could have synergic effect with the steam treatment in the sugar recovery. The use of oxalic acid instead of ammonium oxalate was preferred because acid can catalyze the polymer hydrolysis.

2. MATERIALS AND METHODS

2.1 The biomass

The biomass examined in this study has been the *Zostera marina* harvested in north Germany and provided by Amt-Klutzer, it was submitted for the steam treatment as arrived, although it was evident sand and animal residues (crab fragments, seagull plumes, little shells etc.).

The physical aspect remembers a dark green grass with ribbon shaped leaves 20-50 cm in length.

Batches of 200g, humidified with 250g of aqueous solution, were loaded in the steam reactor.

2.2 Choosing the experimental variables

The experimental variables that have been investigated are temperature and time of steam treatment and the amount of oxalic acid loaded. By previous experiences, we knew that temperatures higher then 180 °C and treatments longer than 5 minutes are good for hardwood, but too sever when applied to algae. *Vice versa* temperatures below 160 °C and times below 1 minute let a too bland treatment and a mediocre separation of the fibre macro constituents. As regard the loading of oxalic acid, as first level approach, to avoid a high employment of chemicals in the process, it was retained not higher then 2% respect the starting dry biomass.

In synthesis, the effects of the steam explosion treatment and its optimization were explored in the following range:

T (°C)	160-180
Time (sec),	60-300
Oxalic acid (% on dry algae)	0-2

2.3 *Experimental design*

The planning of the experiments was made by a software called “Essential Experimental Design”(‘97). It provides the empiric correlation to the process variables by interpolation equations.

In our case the variables were 3: temperature, time and percent loading of oxalic acid; the minimum centre points were been 2 and the Level were set at higher resolution (full factorial). The runs suggested resulted 10, as they were obtained as combination of the extremes values of the parameters and one central condition to be carried out in duplicate.

It is possible to show the runs as the vertexes of a cube plus two coincident centre point (Figure 1). Table 1 shows the details of the ten treatment conditions.

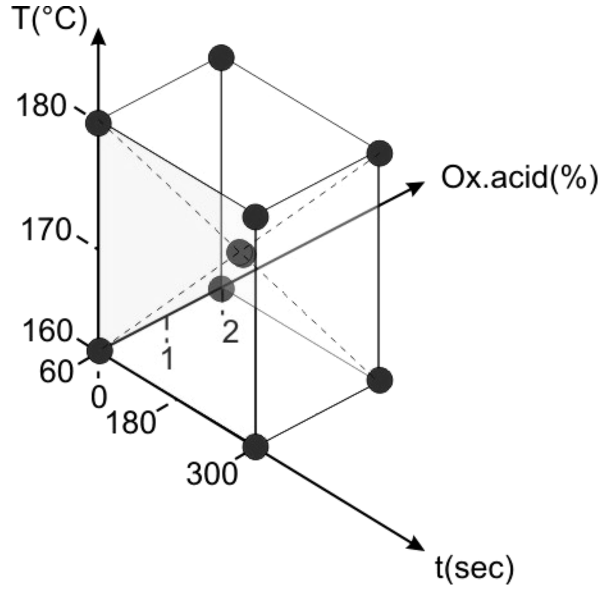


Figure 1 – The vertexes and the centre of the cube indicate the investigated experimental design.

Table 1 – List of the ten experimental runs.

run	T (°C)	time (second)	Oxalic acid per 100g$_{alga}$ (g)
1	180	300	2
2	180	300	0
3	180	60	2
4	180	60	0
5	170	180	1
6	170	180	1
7	160	300	2
8	160	300	0
9	160	60	2
10	160	60	0

The design of the experiment was analyzed by the correlation of the variables, indicates in Table 1, with the experimental answers. The software provides the following equation for empiric interpolation :

$$R = b_0 + b_1 T + b_2 t + b_3 ox + b_4 T \cdot t + b_5 T \cdot ox + b_6 t \cdot ox \qquad \text{[eq. 1]}$$

Where:

R	experimental datum
T	temperature (°C)
t	time (s)
ox	oxalic acid (%)
b_n	coefficients

2.4 Experimental procedure

2.4.1 Sample preparation

For each run, the experimental procedure of steam treatment was carried out following the scheme reported in Figure 2. In particular:

- *Oxalic acid pre-conditioning*

The raw Zostera (about 200 g) was put in a vessel, then an aqueous solution (about 250 g) containing 0, 2, or 4 g of oxalic acid was sprinkled up, while the material was gently stirred for ten minutes.

- *Steam treatment*

After the impregnation step the sample was loaded in the batch steam reactor and treated at the conditions reported in Table 1.
During the steam treatment, the temperature inside the reactor was registered at regular intervals of time.
At the end of the treatment the exploded product was convoyed in the expansion chamber and the procedure repeated to collect enough products.
At last it was completely collected and weighted, then sealed in bags and frozen.

- *Aqueous extraction*

About 100 g of exploded material was extracted two times with 300 ml of hot water (65 °C), then filtered on paper filter WHATMAN 4 using a vacuum pump. The insoluble residue was washed with 100 ml of water, filtered and the resulting solutions were diluted until to obtain 1 litre of aqueous extract. About 200 ml of aqueous extract was evaporated to recover the dried soluble material (SM), while 100 ml was frozen and stored. Both samples were analysed. The insoluble material (IM) was dried overnight at 60 °C, weighted and stored in plastic bags; amounts of 5 g were ground at 50 mesh for the chemical characterization.

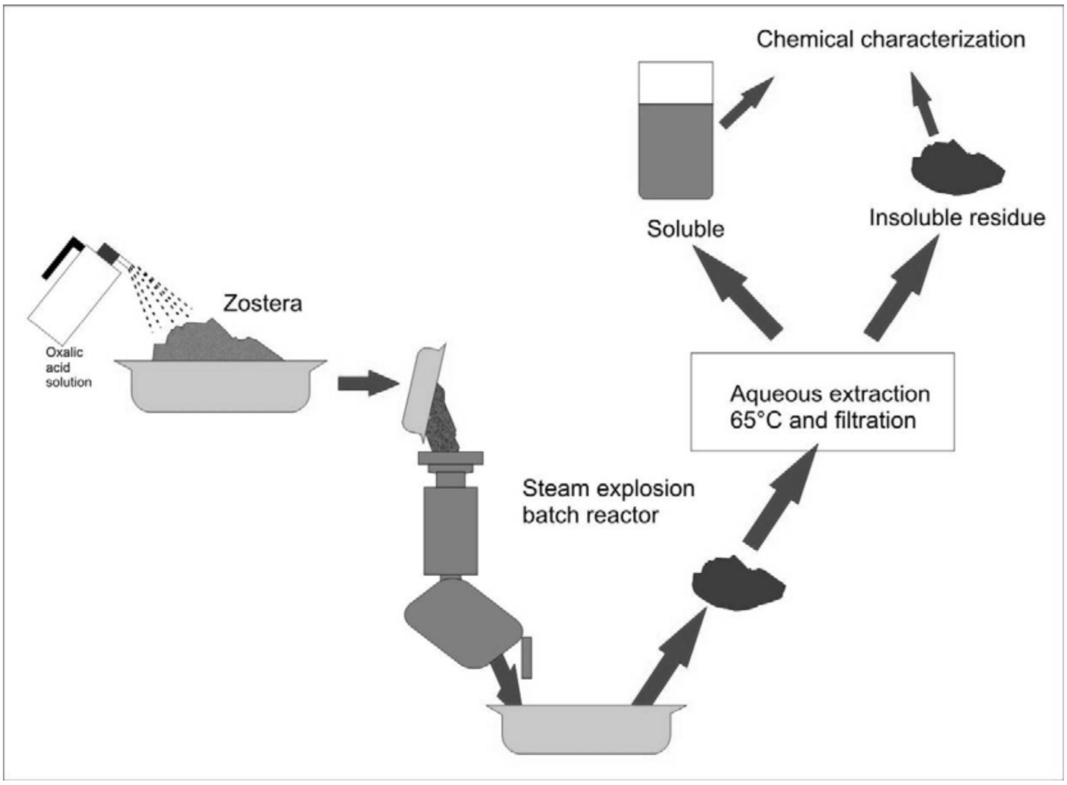


Figure 2 – Procedure for steam explosion and aqueous extraction.

2.5 Chemical characterization

- *Determination of dry matter*

In accordance with a standard procedure for ligno-cellulosic materials, the sample was dried at 105 °C in a thermostatic oven with forced air circulation until its weight remains constant (about 2 hours).

If the sample has to be used for further determinations, it is dried at 60 °C over-night to avoid any degradation that could occur at higher temperatures.

- *Ash determination*

The ash content in the dried, soluble and insoluble, materials was determined by sample combustion at 600 °C (ASTM-D1102, modified). The sample was preliminarily milled at 50 mesh, then dried a 105°C, then it was burnt in the oven raising up the temperature by steps up to 600 °C.

The ash content was determined as percent (w/w) respect the starting material. It is representative of inorganic content of the biomass and it is constituted by a several metal-oxides.

- *Polysaccharides hydrolysis in the insoluble material (IM)*

The primary hydrolysis was carried out on about 0.1 g of dry sample with 1.5 ml of sulphuric acid (72% w/w), using a cone flask, condenser equipped, at room temperature, under shaking for 2 hours. The secondary hydrolysis was carried out diluting the sample with water to 3% w/w acid and boiling the suspension for 4 hours.

After cooling, the acid insoluble residue (AIR) was separated by filtration on glass filter, dried and weighed (Figure 3), (ASTM-D1107 modified method).

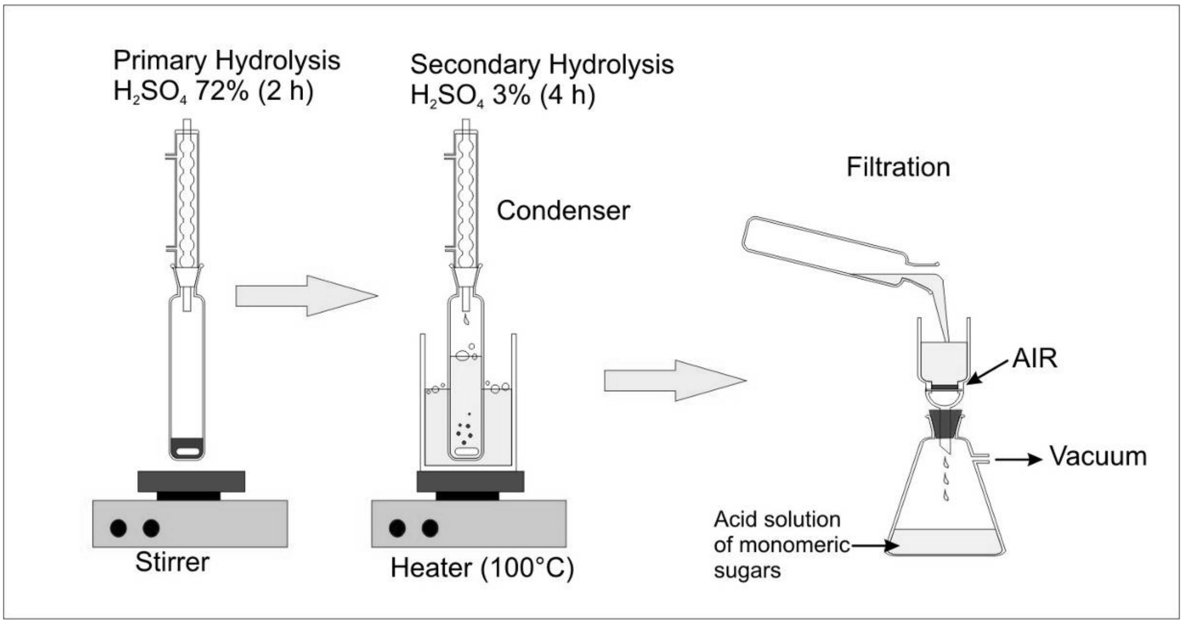


Figure 3 – Acid hydrolysis of the insoluble fraction coming from the SE and extraction procedure.

- *Polysaccharides hydrolysis in the soluble material (SM)*

Polysaccharides in the aqueous extract were hydrolyzed with sulphuric acid 1% at 121 °C in autoclave (P 1 bar), for 1 hour.

About 20 ml of aqueous extract was added with 0.3 ml of sulphuric acid (72%); the solution was put in a plastic tube, hermetically closed, and introduced in autoclave.

- *Sugar determination by HPIC (High Performance Ionic Chromatography*

To simultaneously determine aldoses and uronic acids, a new chromatographic procedure was essayed.

The simultaneously determination of monosacchararides and uronic acids was performed with a Carbopac PA1 column at 28 °C, with a flow rate of 1ml/min, using combined gradient of three eluents: NaOH 200mM, CH_3COONa 1M in 0.1M NaOH, H_2O.

Sugars were eluted by:

- NaOH 200mM: 0-20min, 8mM; 20-21min, 100mM; 21-47min, 80mM; 47-54min, 0mM; 54- 95min, 8mM.
- NaAc 1M in 0.1M NaOH: 21-47min, 250mM, 47-49min, 1M; 49-54min, 1M.

The effluent was monitored using a pulsed-electrochemical detector in the pulsed-amperometric mode, equipped with a gold working electrode and an Ag/AgCl reference electrode, to which potentials of E1 0.1V, E2 0.6V, E3 -0.6V were applied.

Quantification of the samples was performed using a three levels external calibration curve, in the range 2-200 ppm. In Figure 4 are shown two typical chromatograms correspondent to a standard and a real sample.

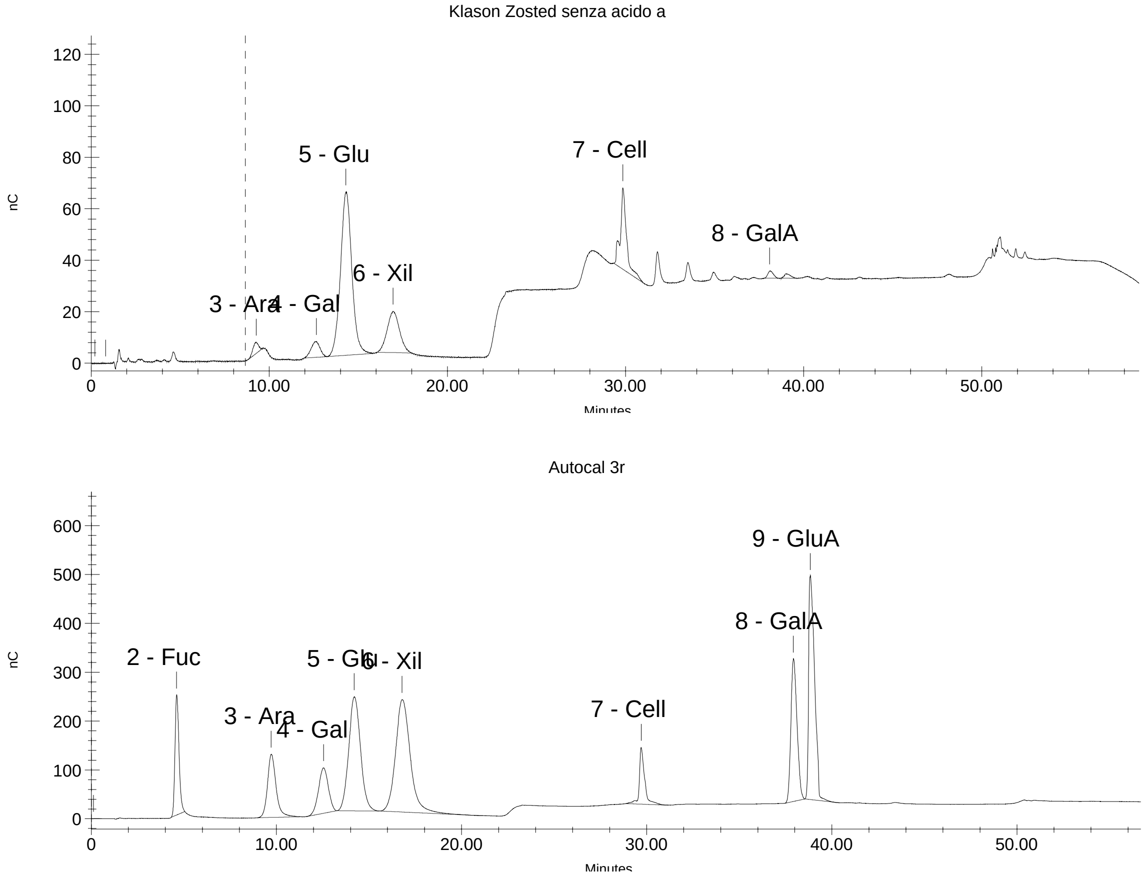


Figure 4 – HPIC chromatograms obtained by the novel analytical method (above: hydrolysates of water insoluble Zostera, below: standard mix).

3. RESULTS

The experimental answers taken to evaluate the treatment effects on Zostera by the steam explosion process (SE), have been:

- Mass recovery after SE;
- Amount of aqueous extract (crude zosterine, SM) obtained washing the exploded product;
- Amount of the sugars detected in the aqueous extract (SM);
- Amount of the sugars detected in the washed exploded (IM);

3.1 Steam explosion treatment

The severity of the steam explosion treatment was quantified as a function of time and temperature, by the parameter *Ro* (Abatzoglou et al., 1992), (equation 2):

$$Ro = t \cdot \exp\left(\frac{T-100}{14.75}\right) \qquad \text{[eq. 2]}$$

T, temperature (°C)
t, time (minutes)

Higher is Ro, greater are the effects of disruption on biomass fibre.
In the case of the continuous process, the temperature can be assumed constant during the treatment and equation 2 can be correctly applied, but in the case of the batch SE reactor, the temperature can oscillate, especially during the first 30 s, and a more general equation has to be applied to calculate Ro:

$$Ro = \sum_0^N \left[\exp\left(\frac{T_{n+1}-100}{14.75}\right)+\exp\left(\frac{T_n-100}{14.75}\right)\right] * \frac{(t_{n+1}-t_n)}{2} \qquad \text{[eq. 3]}$$

In other words Ro was calculated as the area of the graphs that correlate *exp(/T-100)/14.75* versus *t* (time). Indeed equation 3 is an approximation of equation 4:

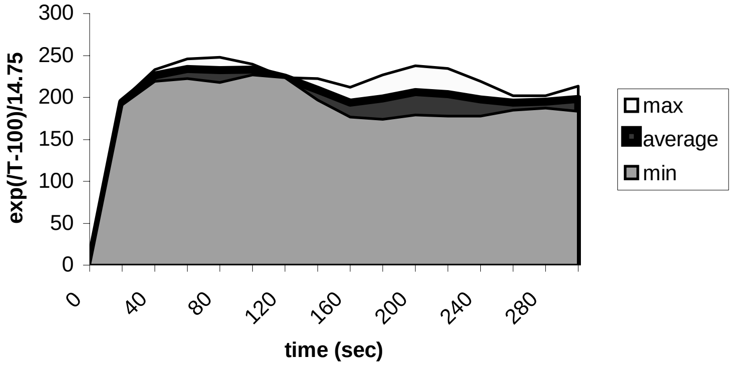
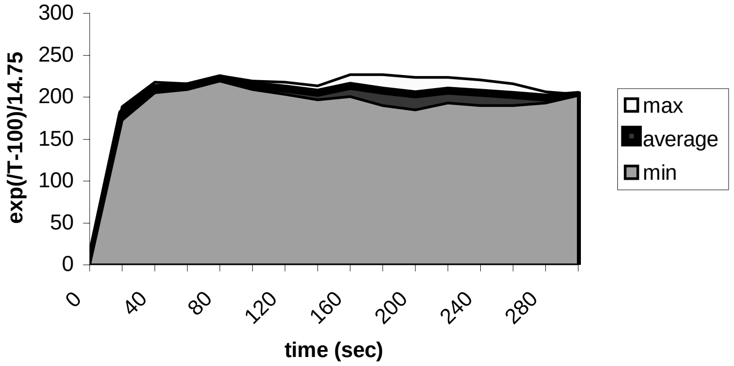
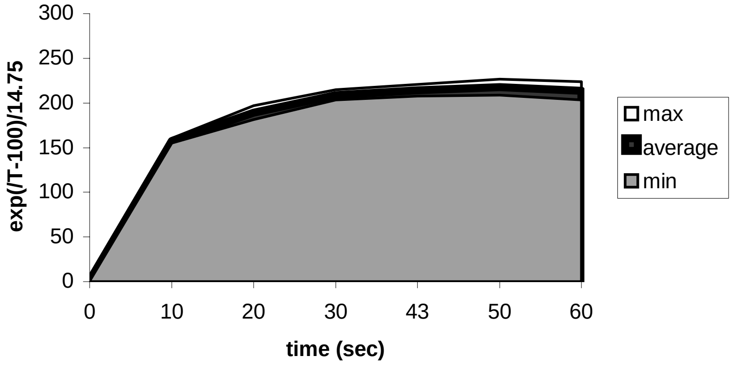
$$Ro = \int_0^t \exp\left(\frac{T-100}{14.75}\right)dt \qquad \text{[eq. 4]}$$

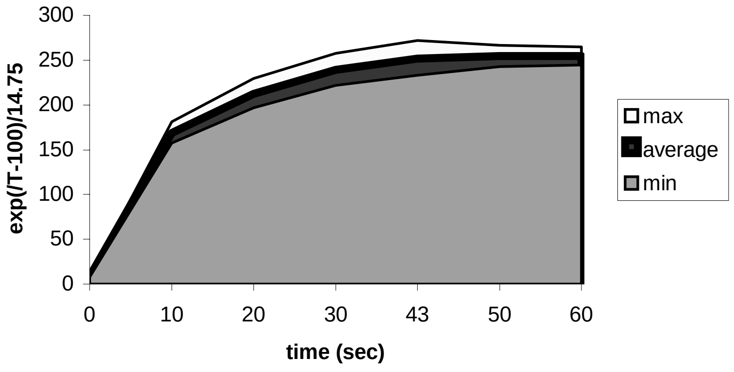
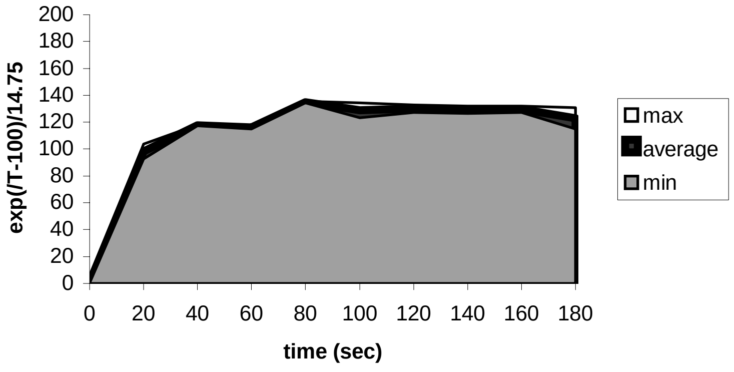
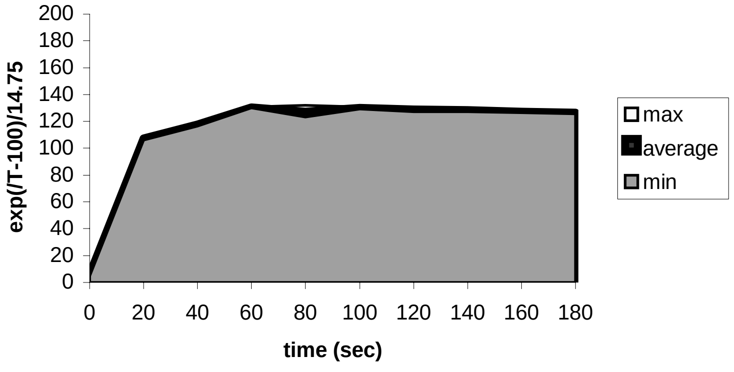
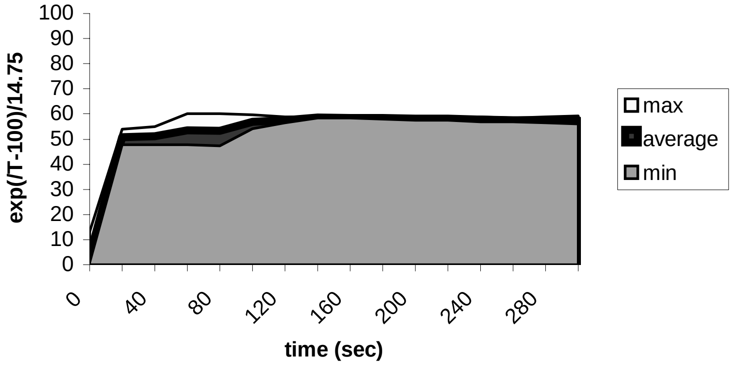
The value of Ro, obtained by equation 3, can be used to calculate an equivalent temperature (T_s) that is temperature to be used to achieve the same severity at stationary condition, such as the continuous process. The equivalent temperature can be calculated using the equation 5:

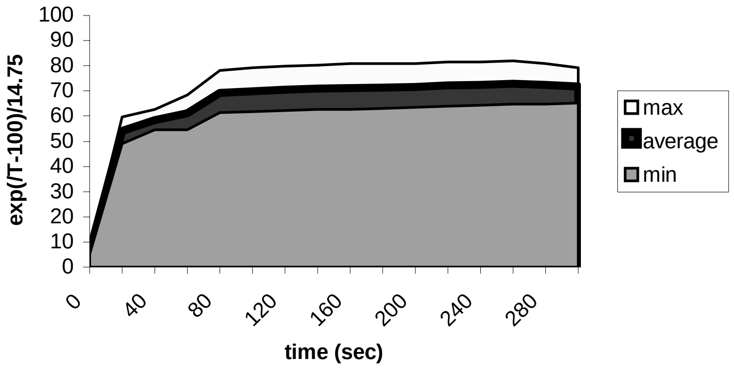
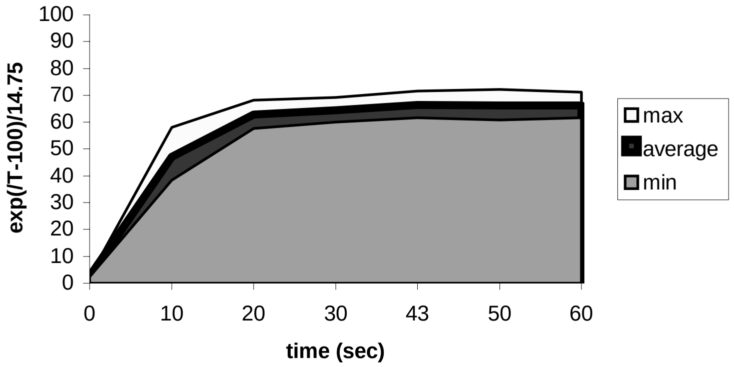
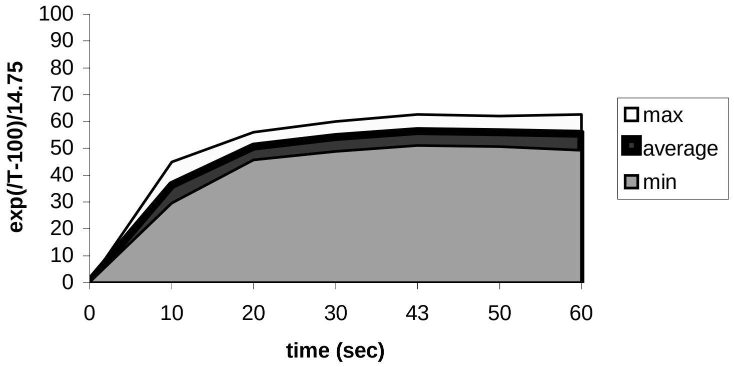
$$T_s = 14.75 * \ln(Ro/t) + 100 \qquad \text{[eq. 5]}$$

In the Table 2 are shown the graphs (correlating *exp(/T-100)/14.75* versus *t)*, the LogRo values calculated by equation 3, and the T_s calculated by equation 5, obtained for the ten treatments.

Table 2 – Data of SE batch reactor for the ten different treatment conditions.

Run	graph	Severity Log Ro	Temperature T_s (°C)
180°C 300 s 2%ox.a.		3.01	178.3 ±1.0
180°C 300 s 0%ox.a.		3.00	178.1 ± 0.7
180°C 60 s 2%ox.a.		2.26	178.1 ± 0.5

180°C **60 s** **0%ox.a.**		2.32	178.8 ± 1.0
170°C **180 s** **1%ox.a.**		2.54	170.2 ± 0.4
170°C **180 s** **1%ox.a.**		2.55	170.4 ± 0.2
160°C **300 s** **2%ox.a.**		2.44	159.0 ± 0.6

160°C **300 s** **0%ox.a.**		2.52	161.9 ± 1.7
160°C **60 s** **2%ox.a.**		1.76	159.6 ± 1.4
160°C **60 s** **0%ox.a.**		1.67	156.8 ± 1.7

From the Table 2 it can be inferred that the difference between the set temperature and T_s does not exceed 2 °C and only in the last run the difference overcomes the 3 °C.

3.2 Mass recovery after SE

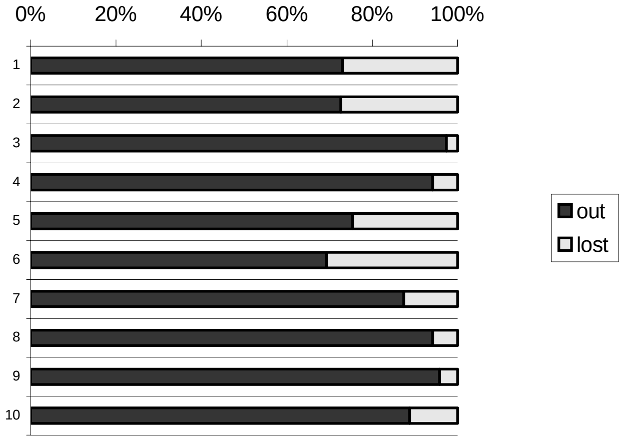
The mass recovery after the SE treatment was determined as ratio between the dry matter produced (out) and the dry matter loaded (in). Generally, the recovery is not quantitative because the treatment causes degradation of the biomass components (at different extent) into volatiles that are lost. Obviously, mass recovery is the primary datum when yields have to be assessed; further, it is required for mass and energy balance.

3.3 Experimental answers

Table 3 shows the mass recovery obtained for each run.

Table 3 – Mass recovery after SE, experimental data.

Run	Mass recovery (%)
180°C,300 s, 2% ox.a.	**73.0**
180°C,300 s, 0% ox.a.	**72.7**
180°C,60 s, 2% ox.a.	**97.4**
180°C,60 s, 0% ox.a.	**94.1**
170°C,180 s, 1% ox.a.	**75.5**
170°C,180 s, 1% ox.a.	**69.3**
160°C,300 s, 2% ox.a.	**87.3**
160°C,300 s, 0% ox.a.	**94.1**
160°C,60 s, 2% ox.a.	**95.7**
160°C,60 s, 0% ox.a.	**88.8**



3.4 Data elaboration

Introducing the mass recovery values as responses in the “Essential Experimental Design”, it was produced the data elaboration. In Table 4 are reported the coefficients obtained for the equation 1, while the Figure 5 shows particular cases of the correspondent graphs.

Table 4 – Coefficients of eq. 1 for mass recovery after SE.

b0	22.61
b1	0.40
b2	0.72
b3	-3.72
b4	-0.004
b5	0.04
b6	-0.02

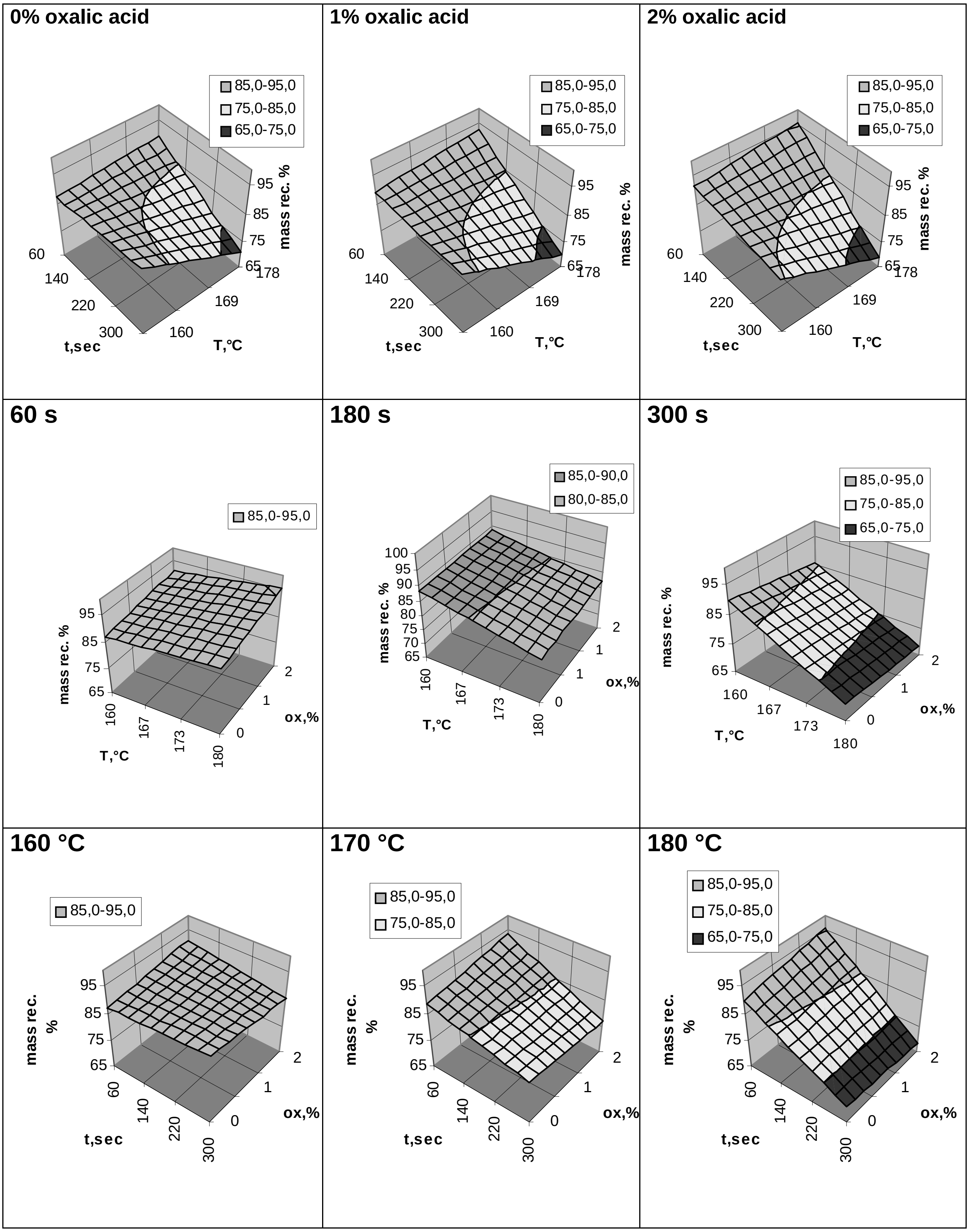


Figure 5 –Mass recovery after SE.

The minimum and maximum values of the mass recovery after SE are reported in Table 5

Table 5 – The min and max predicted values of mass recovery after SE.

Mass recovery (%)		T(°C)	t(sec)	Ox (%)
min	68.6	180	300	2
max	95.6	180	60	2

3.4.1 Considerations

Analyzing the data some considerations can be drawn:

- The effect of oxalic acid has been slight;
- The loss of mass is increased with increasing of time and temperature;
- At low temperature or time, the variation of the other two variables has not had effects;

Our experience says that generally the mass loss (as volatile compounds) after the SE treatment on lignocellulosic biomass, even at high severity, does not exceed 20%. In the case of Zostera we have obtained a mass loss over 30%. This value is relatively high and can be attributed to the weakness of Zostera fiber.

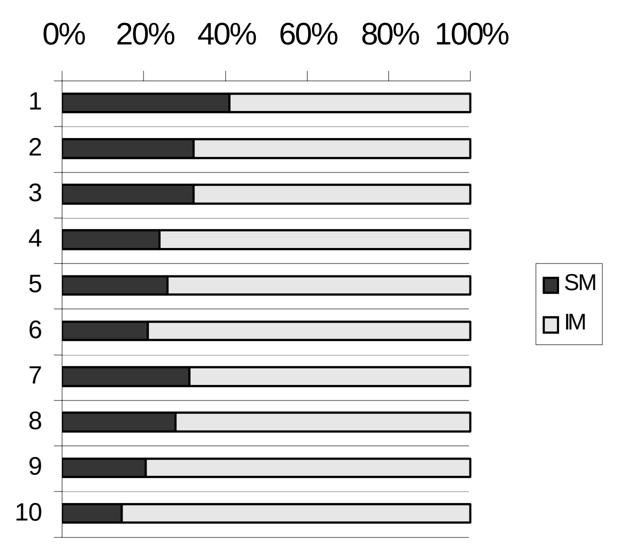
3.5 Aqueous extraction on SE product and soluble material (SM) recovery

The aqueous extraction removes soluble components, generally oligomers freed by SE treatment. The amount of the aqueous extract (soluble material, SM) can be an indication of crude zosterine recoverable. Generally it increases with the treatment severity; the effect of oxalic acid pre-conditioning is resulted effective.

3.5.1 *Experimental answers*

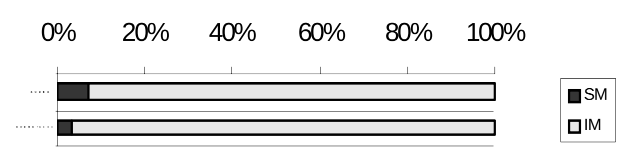
Table 6 shows the value of SM recovery after water washing of the exploded products.

Table 6 – SM recovery after exploded product washing, experimental data. IM = insoluble material.

Run	SM recovery (%)	
180°C,300 s, 2% ox.a.	**40.8**	
180°C,300 s, 0% ox.a.	**32.3**	
180°C,60 s, 2% ox.a.	**32.4**	
180°C,60 s, 0% ox.a.	**23.8**	
170°C,180 s, 1% ox.a.	**25.9**	
170°C,180 s, 1% ox.a.	**21.2**	
160°C,300 s, 2% ox.a.	**31.4**	
160°C,300 s, 0% ox.a.	**27.7**	
160°C,60 s, 2% ox.a.	**20.6**	
160°C,60 s, 0% ox.a.	**14.6**	

It is worth of noting that the extraction performed on the raw material, also oxalic acid preconditioned, was been very lowly efficient, as reported in Table 7. Indeed SE is resulted a good pretreatment to efficiently fractionate the studied biomass.

Table 7 – SM recovery after raw material extraction, also oxalic acid pre-conditioned.

sample	SM recovery (%)	
raw 0% ox.a	**7.3**	
raw 2% ox.a	**3.4**	

3.5.2 *Data elaboration*

Introducing the SM recovery values as responses in the "Essential Experimental Design", it was produced the data elaboration. In Table 8 are reported the coefficients obtained for the equation 1, while the Figure 6 shows particular cases of the correspondent graphs.

Table 8 – Coefficients of eq. 1 and correlation index for SM recovery after washing of SE product.

b0	-64.8
b1	0.47
b2	0.17
b3	-11.8
b4	-0.0007
b5	0.09
b6	-0.002

$R^2 = 0.915$

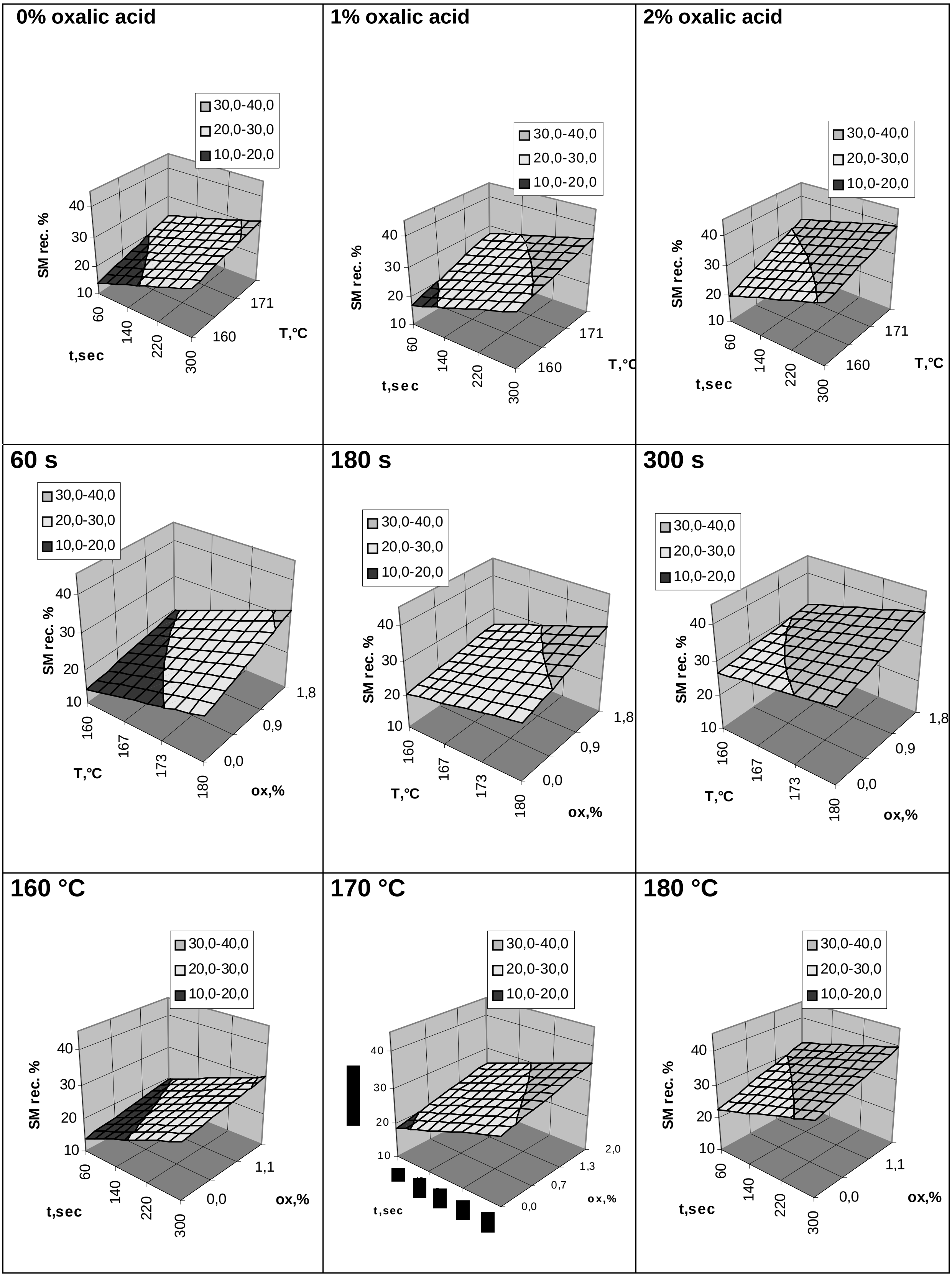


Figure 6 –Soluble Matter recovery after SE product washing.

The minimum and maximum values of the SM recovery are reported in Table 9.

Table 9 – The min and max predicted values of SM recovery.

SM recovery (%)		T(°C)	t(sec)	Ox (%)
min	14.0	160	60	0
max	39.6	180	300	2

3.5.3 *Considerations*

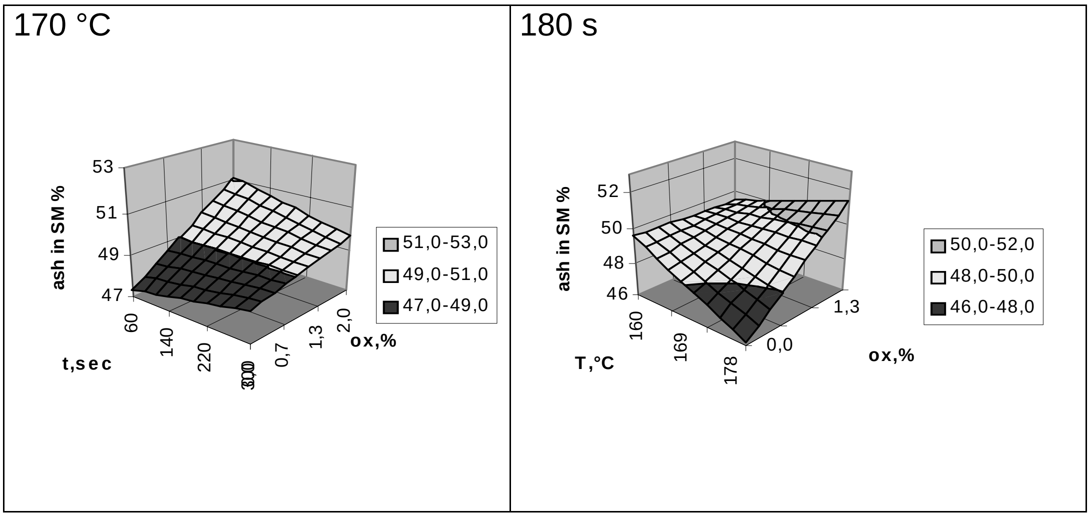
Soluble Matter recovery (after extraction of the SE products) can give indications on the crude zosterine extractability. In the explored range, the SM recovery is varied between 14 and 40 % and a considerable contribute is given by oxalic acid that at high temperature has increased the SM by 8 percent units, while at low temperature by 5 percent units. Indeed oxalic acid has opened the fibre, making it more hydrolysable, by sequestering the incorporated cations.

As confirmation, an interesting aspect was been the repartition of inorganic between SM and IM. These data, obtained by ash determination in SM and IM, are reported in Table 10.

Table 10 – Distribution of total ash after extraction.

	Repartition of total ash (%)	
	In SM	In IM
180°C,300 s, 2% ox.a.	50	50
180°C,300 s, 0% ox.a.	45	55
180°C,60 s, 2% ox.a.	57	43
180°C,60 s, 0% ox.a.	52	48
170°C,180 s, 1% ox.a.	41	59
170°C,180 s, 1% ox.a.	41	59
160°C,300 s, 2% ox.a.	53	47
160°C,300 s, 0% ox.a.	56	44
160°C,60 s, 2% ox.a.	50	50
160°C,60 s, 0% ox.a.	47	53

Although the inorganic content is resulted almost equal-distributed, higher values of ash percent are determined in SM derived from 2% oxalic acid preconditioned algae. The graphs of Figure 7, obtained by "Essential Regression", show better the oxalic acid effect on increasing ash (metals) in SM.


170 °C

180 s

Figure 7 – Oxalic acid influence in the distribution of ash after SM extraction (2 cases).

3.6 Amount of sugars recovered in the aqueous extract

The recovery of sugars by steam explosion and aqueous extraction, can be considered one of the ways to valorise algae.

3.6.1 *Experimental answers*

On aqueous extract, acid hydrolysis was performed and sugars were detected by HPIC. Table 11 reports the percentage of sugars respect to the starting exploded substrate (ES).

Table 11 – Sugar recovery in the aqueous extract, referred to the exploded Zostera.

run	sugar/ES, %							
	arabinose	xylose	galactose	fucose	glucose	GalA	GluA	total
180°C,300 s, 2% ox.a.	0.61	1.84	0.80	0.10	0.74	0.85	0.12	**5.1**
180°C,300 s, 0% ox.a.	0.32	1.26	0.58	0.08	0.42	0.50	0.09	**3.3**
180°C,60 s, 2% ox.a.	0.29	1.00	0.49	0.09	0.80	1.57	0.08	**4.3**
180°C,60 s, 0% ox.a.	0.26	0.55	0.43	0.09	0.70	0.35	0.06	**2.4**
170°C,180 s, 1% ox.a.	0.46	0.97	0.58	0.18	0.49	0.75	0.09	**3.5**
170°C,180 s, 1% ox.a.	0.46	0.93	0.58	0.14	0.35	0.61	0.08	**3.2**
160°C,300 s, 2% ox.a.	0.53	1.18	0.59	0.13	0.82	1.61	0.10	**5.0**
160°C,300 s, 0% ox.a.	0.28	0.62	0.35	0.07	1.10	0.31	0.05	**2.8**
160°C,60 s, 2% ox.a.	0.25	0.48	0.31	0.07	0.69	0.56	0.05	**2.4**
160°C,60 s, 0% ox.a.	0.06	0.14	0.16	0.03	0.85	0.14	0.02	**1.4**

3.6.2 *Data elaboration*

Introducing the total sugar values as responses in the DOE software, the coefficients were obtained for the equation 1 (Table 12); the Figure 8 shows the correspondent graphs.

Table 12 – Coefficients of eq. 1 and correlation Index for sugar recovery in SM.

b0	-12.26
b1	0.082
b2	0.047
b3	-0.47
b4	-0.0002
b5	0.007
b6	0.001

$R^2 = 0.978$

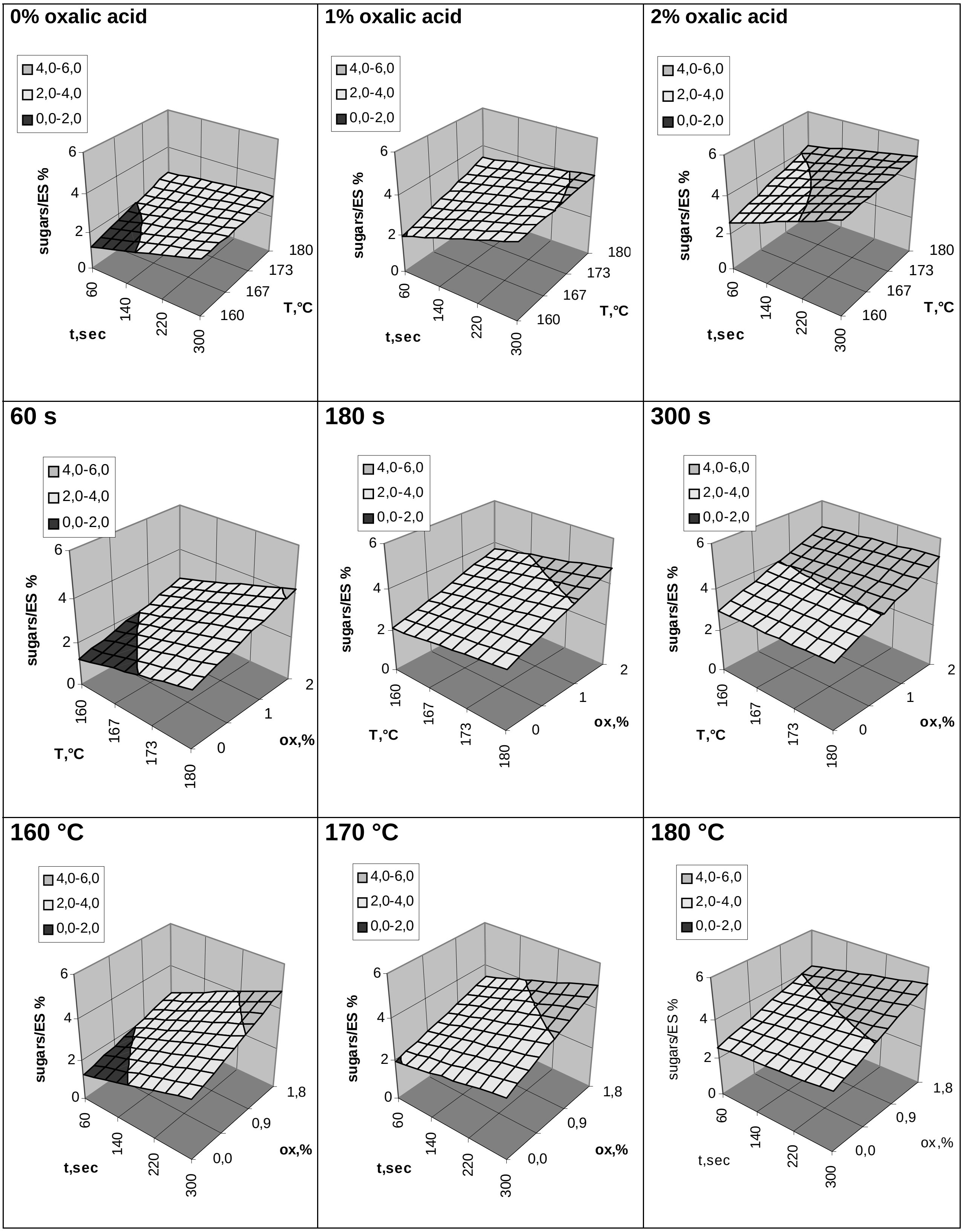


Figure 8 –Sugars recovery in the aqueous extract.

The minimum and maximum values are reported in Table. 13.

Table 13 – The min and max predicted values of sugar recovery in the SM.

Sugars/ES (%)		T(°C)	t(sec)	Ox (%)
min	1.25	160	60	0
max	5.2	180	300	2

3.6.3 *Considerations*

Extraction performed on Zostera raw material allowed a slight sugars recovery of 0.5g/100 g, by preconditioning with oxalic acid the yield did not increase. The SE treatment increased the recovery yields.

As can be inferred by analyzing the slopes of the interpolating curves, the major contribute to increase the ratio sugars/ES is provided by the oxalic acid which load from 0 to 2% has increased the value on average 74% at constant T and t (Table 14)

Table 14 –Effect of the oxalic acid pre-impregnation on the sugar extraction.

	Sugars/ES (%) predicted values		
	0% ox.a.	**2% ox.a.**	**Δ%**
160 °C, 60 s	1.3	2.6	100
180 °C, 60 s	2.6	4.2	62
160 °C, 300 s	2.9	4.8	66
180 °C, 300 s	3.1	5.2	68
average			**74**

Data about sugar recovery have been discussed also analyzing the sugars/SM ratio. In this case, the positive effect of oxalic acid has been more evident (Figure 9).

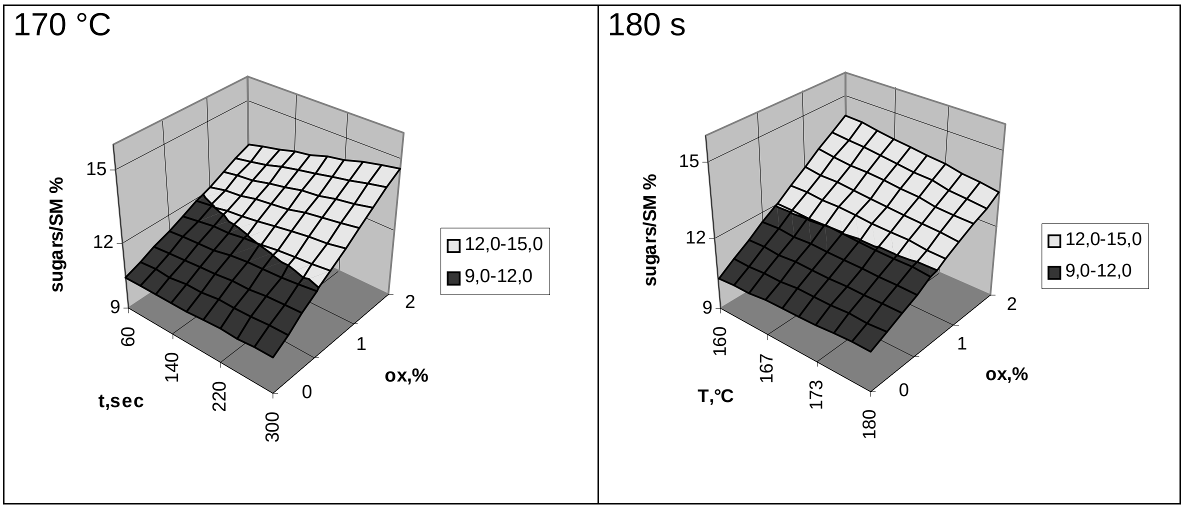


Figure 9 – Efficiency of oxalic acid to enhance sugars/SM ratio.

The amount of the free sugars/SM in the aqueous extract, even is resulted larger at high severity, has not been higher then 4.1 %.

Sugars detected in the aqueous extract are resulted low and over the 60% of the IM is unknown. To identify other substances IR spectra have been carried out.

3.6.4 *IR Spectra of SM*

- *Method*

The dried SM was used to prepare KBr disc for the IR spectra recording. The Spectra were collected on a Perkin Elmer 2000 FTIR spectrometer in the region 4000 ÷ 650 cm^{-1}. The spectral resolution was 2 cm^{-1}. Each spectrum resulted from the average of 16 acquisitions; no elaborations were performed on it.

- *Results*

The comparison of the spectra acquired on the several samples shows that there are no significant differences among the solid residues of the extractions.

This result suggests that the severity of the conditions explored with the SE experiments has no different consequence on the properties of the material extracted.

3.7 ***Sugars residual in the washed exploded (IM)***

The insoluble material, derived from the exploded product washing, was hydrolysed with acid to depolymerise the material and quantify monomeric sugars.

Two fractions were obtained:

1. hydrolyzed sugar solution;
2. solid dark residue (acid insoluble residue (AIR).

The interest to examine the sugar content of IM comes from the possibility to convert it into biofuel, such as ethanol. The ratio sugars/IM was then determined and results were analyzed by the "Essential Experimental Design" as above described.

3.7.1 *Experimental answers*

The sugar content of hydrolyzed solution, deriving from each IM, was determined by HPIC. The percentage of the sugars, calculated to the starting IM, is reported in Table. 15.

Table 15 –Sugars content in the Insoluble Matter of exploded Zostera.

	% sugar/IM				
	arabinose	xylose	galactose	glucose	total
180°C,300 s, 2% ox.a.	0.10	1.39	0.80	28.10	**30.4**
180°C,300 s, 0% ox.a.	0.15	1.79	0.95	26.55	**29.4**
180°C,60 s, 2% ox.a.	0.20	2.43	1.55	21.04	**25.2**
180°C,60 s, 0% ox.a.	0.11	2.53	1.57	17.53	**21.7**
170°C,180 s, 1% ox.a.	0.23	2.85	0.69	13.46	**17.2**
170°C,180 s, 1% ox.a.	0.24	1.96	1.33	13.02	**16.6**
160°C,300 s, 2% ox.a.	0.00	2.35	0.64	26.74	**29.7**
160°C,300 s, 0% ox.a.	0.00	2.63	0.70	22.69	**26.0**
160°C,60 s, 2% ox.a.	0.00	2.75	0.80	21.49	**25.0**
160°C,60 s, 0% ox.a.	0.00	3.08	0.88	21.09	**25.1**

3.7.2 *Data elaboration*

Introducing the total sugar values as responses in the "Essential Experimental Design", it was produced the data elaboration. In Table 16 are reported the coefficients obtained for the equation 1.

Table 16 – Coefficients of eq. 1 for sugar in IM.

b0	42.8
b1	-0.13
b2	-0.11
b3	-0.76
b4	0.0008
b5	0.009
b6	0.001

The correlation index did not result significant. The correlation has failed because in the center point samples too low sugars content is detected. Also, there has been evidence of probable incomplete hydrolysis of sugar in the chromatogram showed in Figure 4 (sample): after the cellobiose peak, in the range 32-38 minutes, peaks of unknown can be pointed out, probably they are oligomers at increasing polymerization degree. Then sugar could be underestimated, even though acid hydrolysis procedure was optimized for the raw material: weak or strong acid hydrolysis then the employed procedure has indeed produced less free sugars (Figure 10). Acid hydrolysis to quantify total sugar has to be yet optimized.

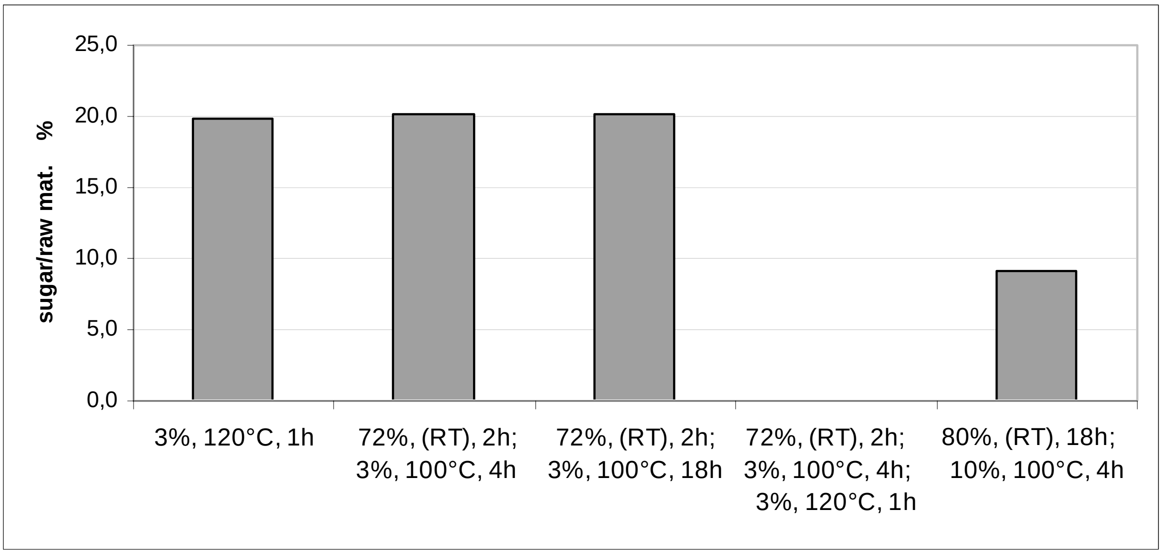


Figure 10 – Efficiency of different procedure for the acid hydrolysis of Zostera
(Legend: sulfuric acid concentration w/w, temperature of hydrolysis, treatment time)

A part any discussion about the hydrolysis efficiency, it should be stressed that the high glucose content (up to 28% in IM) make the substrate interesting to ethanol bioconversion processes.

3.8 Acid insoluble residue (AIR) in the washed exploded (IM)

The AIR content should be complementary to the sugar content. In the case of terrestrial plants, SE does not affect the lignin content, but in any cases, at high severity, lignin can be partially degraded passing in acid solution. To have a wider overlook also AIR was investigated.

3.8.1 <u>Experimental answers</u>

AIR resulting from each IM was weighted and reported as percentage to IM (Table 17).

Table 17 – Acid Insoluble Residue content in the aqueous Insoluble Matter of exploded Zostera.

	AIR/IM, %
180°C,300 s, 2% ox.a.	**34.2**
180°C,300 s, 0% ox.a.	**39.4**
180°C,60 s, 2% ox.a.	**28.3**
180°C,60 s, 0% ox.a.	**24.9**
170°C,180 s, 1% ox.a.	**30.1**
170°C,180 s, 1% ox.a.	**30.3**
160°C,300 s, 2% ox.a.	**28.5**
160°C,300 s, 0% ox.a.	**31.1**
160°C,60 s, 2% ox.a.	**24.9**
160°C,60 s, 0% ox.a.	**23.9**

3.8.2 Data elaboration

Introducing the value of AIR/IM as responses in the "Essential Experimental Design", it was produced the data elaboration. In Table 18 are reported the coefficients obtained for the equation 1, while Figure 11 shows particular cases of the correspondent graphs.

Table 18 – Coefficients of eq. 1 and correlation index for sugar in IM.

b0	12,6	
b1	0,05	
b2	-0,12	R^2 = 0.979
b3	2,08	
b4	0,001	
b5	-0,001	
b6	-0,01	

The minimum an maximum values are reported in Table 19.

Table 19 – The min and max predicted values of AIR in the IM.

	AIR/IM (%)	**T(°C)**	**t(s)**	**Ox (%)**
min	23.4	160	60	0
max	39.0	180	300	0

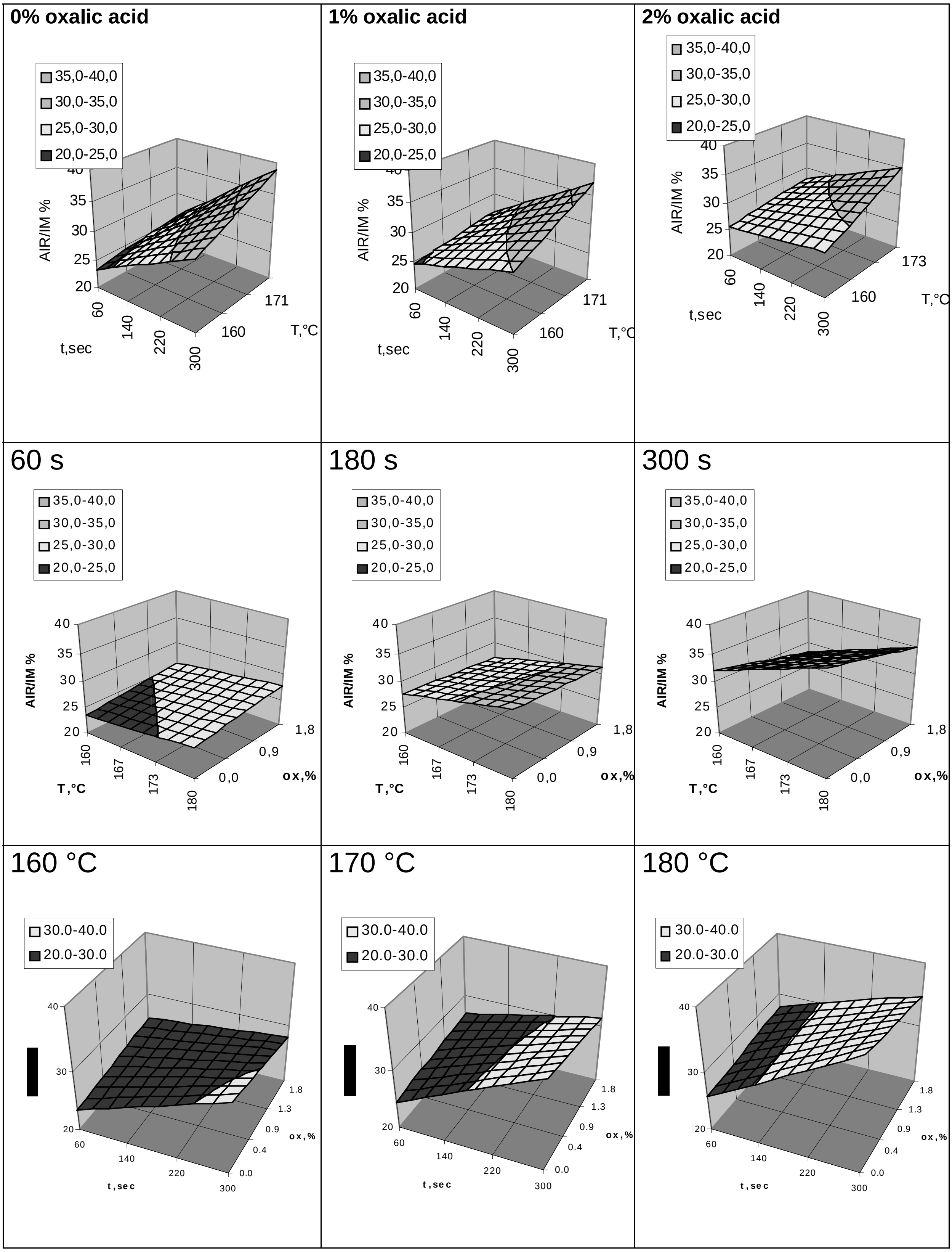


Figure 11 –Acid Insoluble Residue content in the aqueous Insoluble Matter of exploded Zostera.

3.8.3 *Considerations*

AIR increased with the severity and oxalic acid slightly affected it.

The higher values at high severity can be considered as consequence of major SM extraction and correspondent IM reduction: being AIR not water extractable, the ratio AIR/IM is increased.

3.9 Summarized data

In Table 20 are summarized the data concerning the optimization of extracting exploded Zostera.

Table. 20 – Summarized data of zoostera treatments.

	%								
	ES/Raw	IM/ES	SM/ES	Su/IM	Su/SM	mSu/SM	Ash/IM	Ash/SM	AIR/IM
180°C,300 s, 2% ox.a.	73.0	59.2	40.8	30.4	12.4	2.3	17.7	26.0	34.2
180°C,300 s, 0% ox.a.	72.7	67.7	32.3	29.4	10.1	1.2	17.0	29.0	39.4
180°C,60 s, 2% ox.a.	97.4	67.6	32.4	25.2	13.4	2.9	11.4	31.2	28.3
180°C,60 s, 0% ox.a.	94.1	76.2	23.8	21.7	10.2	3.0	10.8	36.8	24.9
170°C,180 s, 1% ox.a.	75.5	74.1	25.9	17.2	13.6	2.0	17.4	34.3	30.1
170°C,180 s, 1% ox.a.	69.3	78.8	21.2	16.6	14.9	1.8	13.8	35.8	30.3
160°C,300 s, 2% ox.a.	87.3	68.6	31.4	29.7	15.8	2.4	11.8	29.4	28.5
160°C,300 s, 0% ox.a.	94.1	72.3	27.7	26.0	10.0	4.1	10.8	36.2	31.1
160°C,60 s, 2% ox.a.	95.7	79.4	20.6	25.0	11.6	0.0	10.1	38.6	24.9
160°C,60 s, 0% ox.a.	88.8	85.4	14.6	25.1	9.6	0.6	9.0	46.7	23.9
Raw 0% ox.a.	-	92.7[a]	7.3[a]	22.4	6.2	0	11.3	54.3	24.2
Raw 2% ox.a	-	96.6[a]	3.4[a]	21.2	7.4	0	10.5	52.5	27.0

Raw = raw material (untreated Zostera); ES = exploded substrate (steam explosion product); IM = insoluble material after ES water extraction; SM = soluble material after ES water extraction; Su = total sugars detected after acid hydrolysis; mSu = monomeric sugars in not hydrolyzed SM; AIR = acid insoluble residue after acid hydrolysis; [a] Raw instead of ES.

III: Characterization of Products and Byproducts from Steam Explosion

1. APPROACH

As first step, it has been checked if the standard methods used to analyse the lignocellulosic plans were applicable to analyse the raw and steam exploded Zostera and Ulva.

The aim has been to report the composition of the materials in terms of ash, extractives, crude zosterine, carbohydrates (*rhamnose, glucose, xylose, arabinose, mannose, galactose, galacturonic acid, glucuronic acid, fucose*). Elemental analysis has also been carried out.

After the SE, the biomass has been extracted with warm water. The soluble and insoluble fractions have been assessed as mass recovery; the soluble sugars in the aqueous extract were analysed by HPIC (High Performance Ionic Chromatography).

The aqueous extract has been hydrolysed by strong acid to break down the soluble oligosaccharides, the resulting monomer sugars have been determined by HPIC.

The water insoluble residue has been hydrolysed both by strong acid and enzymes (cellulase) to compare the glucose production yield. The recovery of the constituents has been reported as a function of the SE severity.

2. EXPERIMENTAL

The scheme of treatment and characterization is reported in Figure 1, where streams and experimental methods are reported, the single steps are detailed in the following.

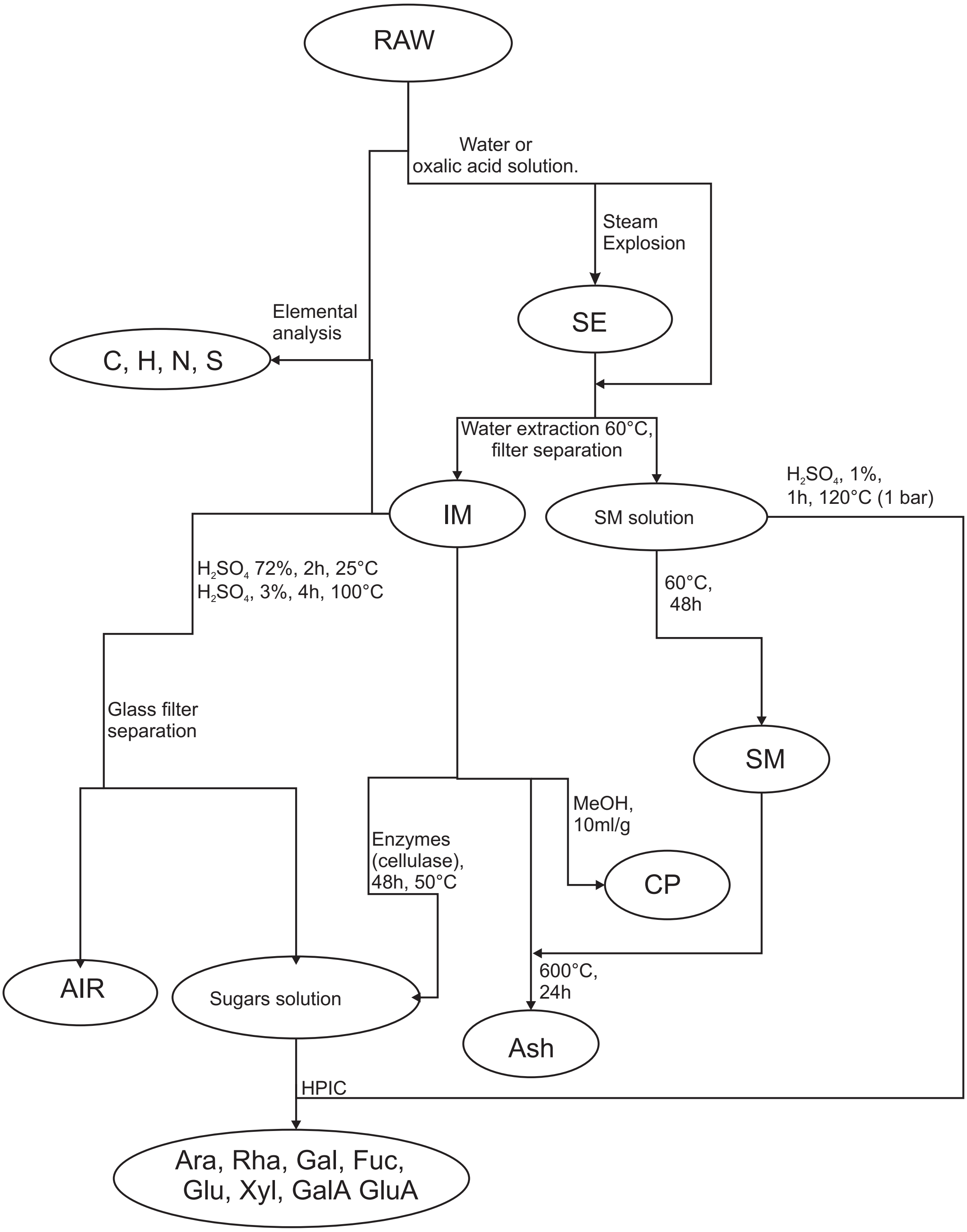


Figure 1 – Scheme of treatment and stream characterization: RAW = raw material (Zostera and Ulva), ES = exploded substrate, IM = aqueous Insoluble Matter, SM = aqueous Soluble Matter, AIR = Insoluble Matter after Acid Hydrolysis, CP = Crude Pigment, Ara = arabinose, Rha = rhamnose, Gal = galactose, Fuc = fucose, Glu = glucose, Xyl = xylose, GalA = galacturonic acid, GluA = glucuronic Acid.

2.1 *Materials*

The materials, *Zostera marina* and *Ulva armoricana,* were steam exploded as received (without washing) after adjusting the moisture content to 50%.
In the case of Zostera a pre-impregnation step with oxalic acid was carried out. The raw Zostera (about 200 g) was put in a vessel, then an aqueous solution (about 250 g) containing 0, 2, or 4 g of oxalic acid was sprinkled up, while the material was gently stirred for ten minutes.

2.2 *Steam explosion treatment and water extraction*

The biomass was loaded in the SE batch reactor and treated at the reported conditions (Table 1).
The products were collected and homogenized, and then an amount of 100 g was extracted two times with 300 ml of warm water (65 °C).
The aqueous phase containing the soluble material (SM) was separated from the (solid) insoluble material (IM) by filtration on paper filter, using a water vacuum pump.
The IM was dried overnight at 60 °C and stored in plastic flask.
The solution containing SM was put in plastic flask and frozen.

Table 1 – Steam explosion conditions.

	T (°C)	t (sec)
Zostera	160	60
	160	300
	170	180
	180	60
	180	300
Ulva	150	180
	160	180
	170	180

2.3 *Chemical characterization*

2.3.1 Dry matter

In accordance with a standard procedure for ligno-cellulosic materials, the sample (1-3g) is dried at 105 °C in a forced air circulation oven until constant weight; usually it takes about 2 hours. If the sample has to be used for further determinations, it is dried over-night at 60 °C to avoid any degradation.

2.3.2 Ash determination

The ash content in the dried, soluble or insoluble materials, was determined by sample combustion at 60 0°C in air (ASTM-D1102, modified).

The sample was milled at 50 mesh, dried a 105 °C, then it was burned in the oven by slowly raising the temperature up to 600 °C.

The ash content is reported as percent (w/w) respect the starting material DM (105 °C).

2.3.3 *Pigment extraction*

The treatment with cold methanol (10 ml/$g_{\text{dried sample}}$) was used as method to extract fats, apolar compounds and chlorophyll pigments [2, 23]. The procedure started by adding 10 ml of methanol to 1 g of sample (IM), the suspension was stirred for 3 minutes at room temperature. The liquid phase was separated by centrifugation. The solid residue was washed with 2ml of methanol, the suspension was centrifuged and the liquid phase was added at the previous extract. The methanol solution, green coloured, was dried under vacuum and the solid residue was weighted.

The material extracted with methanol was referred as crude pigment and determined as percent of the starting material (DM 105 °C).

2.3.4 *Elemental Analysis*

The determinations were performed by the CHNS/O Analyzer 2400 Perkin Elmer.

The dry sample is grinded and sieved at 50 mesh then it is introduced in the combustion chamber through a funnel and burned in a pure oxygen environment.

The resulting gases (CO_2, N_2, SO_2 and H_2O) are homogenized, separated in a quartz column containing copper wires and catalysts, then they are detected by a TCD (thermo-conductimeter) detector.

Cysteine was used as external standard. C, H, N, S are reported as percent (w/w) of the starting material (DM 105 °C).

2.3.5 *Hydrolysis of the Polysaccharides in the insoluble material (IM)*

The primary hydrolysis was carried out at room temperature on about 0.1 g of dry sample with 1.5 ml of sulphuric acid (72% w/w), using a cone flask, manually stirring at times for 2 hours. The secondary hydrolysis was carried out diluting the sample with water to 3% w/w acid and boiling the suspension for 4 hours.

After cooling, the acid insoluble residue (AIR) was separated by filtration on glass filter, dried and weighed, (ASTM-D1107 modified method).

2.3.6 *Polysaccharides hydrolysis in the soluble material (SM)*

Polysaccharides in the aqueous extract were hydrolyzed with sulphuric acid at 121 °C in autoclave, for 1 hour. About 20 ml of aqueous extract were added with 0.3 ml of sulphuric acid 72% w/w (the final concentration of the acid was 1%).

The solution was put in a plastic tube, hermetically closed, and introduced in the autoclave.

2.3.7 *Enzymatic hydrolysis of the insoluble material (IM)*

About 1 g of sample (IM) was put in a 50 ml flask and 20.0 ml of a acetate buffer solution (50 mM, pH 4.8) were added. Then a mixture of two commercial enzymes: Celluclast and Novozym (products from Novo Nordisk, Denmark) was added. The amount of enzyme was measured as 60 mg of their protein content, and as internal ratio Celluclast/Novozyme was 6/1. The flask was introduced in an incubator shaker (New Brunswick G25- KC) and shaken at 200 rpm at 50 °C.

The saccharification was stopped after about 48h. 1 ml of sample was withdrawn and 2 ml of $HClO_4$ 1 M were added to denaturise the proteins. The sugar content was determined by HPIC.

2.3.8 Sugar analysis by HPIC

To simultaneously determine aldoses and uronic acids, a new chromatographic procedure, based on the HPIC was essayed.

The monosacchararides and uronic acids were analyzed with a Carbopac PA1 column at 28 °C, with a flow rate of 1ml/min, using combined gradient of: NaOH 200mM, CH_3COONa 1M in 0.1M NaOH, H_2O.

Sugars were eluted by:

- NaOH 200mM: 0-20min, 8mM; 20-21min, 100mM; 21-47min, 80mM; 47-54min, 0mM; 54- 95min, 8mM.
- NaAc 1M in 0.1M NaOH: 21-47min, 250mM, 47-49min, 1M; 49-54min, 1M.

The effluent was monitored using a pulsed-electrochemical detector (ED40) in the pulsed-amperometric mode, equipped with a gold working electrode and an Ag/AgCl reference electrode, to which electrical potentials of 0.1V (E1), 0.6V (E2), -0.6V (E3) were applied. Quantification of the samples was performed using a three-level external calibration curve, in the range 2-200 ppm.

3. RESULTS

3.1 Main process streams

In Table 2 are reported the recovery in the main streams coming from the SE treatment and aqueous extractions with warm water or methanol. The ash content of IM and SM are also reported. (The stream labelled as AIR is the insoluble residue obtained from the acid hydrolysis.)

Table 2 – Recovery yields of Ulva and Zostera after Steam Explosion and aqueous fractionation.

Treat. Condidtions (T, minutes, ox. acid*)		ES/RAW (%)	IM/ES (%)	SM/ES (%)	Ash/IM (%)	Ash/SM (%)	CP/IM (%)	AIR/IM (%)
Ulva	untreated		79,7[a]	20,3	39,4	70,7	4,0	18,3
	150°C, 3'	89,6	72,4	27,6	38,9	56,5	4,5	30,0
	160°C, 3'	84,9	69,5	30,5	39,0	49,5	4,5	28,9
	170°C, 3'	65,5	57,0	43,0	42,3	56,6	3,4	39,1
Zostera	untreated		92,7[a]	7,3	11,3	54,3	1,0	24,2
	untreated, 2%		96,6[a]	3,4	10,5	52,5	1,6	27,0
	160°C,1'	88,8	85,4	14,6	9,0	46,7	0,9	23,9
	160°C,1', 2%	95,7	79,4	20,6	10,1	38,6	1,2	24,9
	160°C,5'	94,1	72,3	27,7	10,8	36,2	1,1	31,1
	160°C,5', 2%	87,3	68,6	31,4	11,8	29,4	1,7	28,5
	170°C,3', 1%	69,3	78,8	21,2	13,8	35,8	1,1	30,3
	170°C,3', 1%	75,5	74,1	25,9	17,4	34,3	1,2	30,1
	180°C,1'	94,1	76,2	23,8	10,8	36,8	0,9	24,9
	180°C,1', 2%	97,4	67,6	32,4	11,4	31,2	2,0	28,3
	180°C,5'	72,7	67,7	32,3	17,0	29,0	2,6	39,4
	180°C,5', 2%	73,0	59,2	40,8	17,7	26,0	3,6	34,2

RAW = raw material, ES = exploded substrate, IM = aqueous insoluble material, SM = aqueous soluble material, AIR = insoluble material after acid hydrolysis, CP = crude pigment.

** oxalic acid/raw material, w/w.*

[a] *IM is relative to RAW instead of ES*

3.1.1 *Highlights*

- As expected the ratio ES/RAW decreases with the increasing of the steam treatment severity, because of the more volatile substances formation (and its lost) at high temperature and time. A standard deviation of 10% should be associated to each measurement owing to the difficult manual recovery of the material in the receiving tank after the SE .

- The ratio IM/ES indicates the insoluble residue recoverable by warm water extraction of the treated material. It decreases with the SE severity. It appears that the oxalic acid improve the water solubility after the SE.

- The water solubility after SE is expressed by the ratio SM/ES (complementary to IM/ES) increases with severity. Oxalic acid acts synergistically with the SE treatment: the acidity and the capacity of metal capture let the material to be more free and soluble (Figure 2).

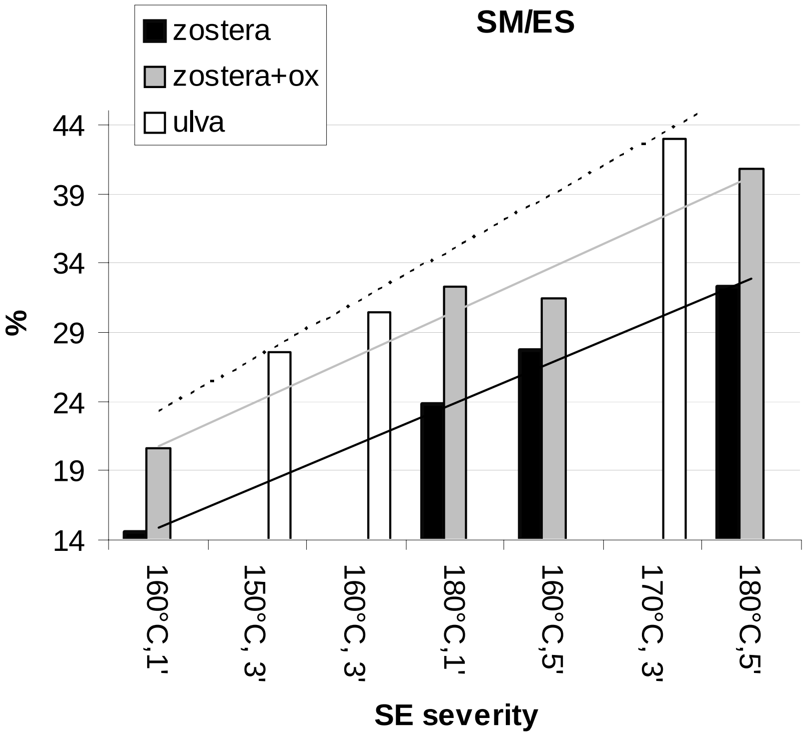


Figure 2 – Water soluble material (SM) of the Steam exploded Zostera and Ulva (Zostera + ox = oxalic acid pre-impregnated Zostera).

- In the Ulva case, the ash content is very high, probably because of the marine salts and sand in the starting material. By washing the Ulva with cold water, the material was “purified” and the ash content decreases to about 20%. With the increasing of the SE severity the ratio Ash/IM seems to increase as well, while the ratio Ash/SM decreases; however, this trend is apparent, indeed the repartition of the ashes, reported in Figure 3, shows that, at high severity, more inorganic material becomes soluble in hot water. In the case of the Zostera Ash/ES increases with the severity and the oxalic acid effect was slight. This is expected because ashes derive from inorganic material, not destroyable by SE.

- Crude pigment (CP), obtained by methanol extraction, increases only in the case of Zostera at high severity. Probably the extraction involves also organic molecules al low molecular weight deriving from SE treatment.

- The insoluble residue deriving from the sulphuric acid hydrolysis (AIR) increases with the severity, but this increasing is relative, not absolute, because AIR is not extractable with hot water and the IM is enriched of it.

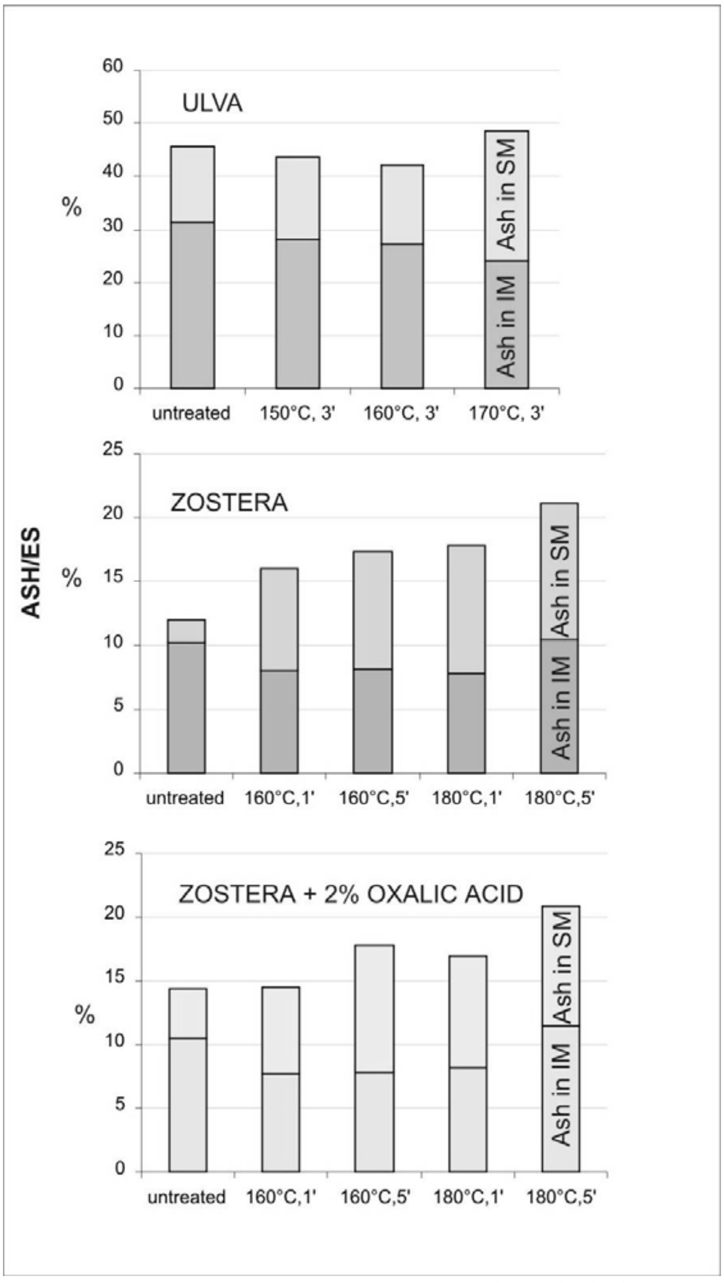


Figure 3 - Ash content in the raw and exploded Zostera: phase's distribution.

3.2 Elemental analysis

In Table 3 are reported the main elements determined on the raw the insoluble material (IM).

In general, small differences between the samples are observed. More interesting is the C/H ratio that increases with the severity. As effect of major sugars extractability with the severity, the ratio goes from typical sugars value (C/H = 6) to more complex structural value like the lignin one (C/H ≈ 10), where aromatic groups are present; indeed AIR increases as above reported.

Table 3 – Elemental analysis of the raw and Insoluble Matter (IM) of Ulva and Zostera as percent w/w.

	IM	**C**	**H**	**N**	**S**	**C/H**
Ulva	untreated	27,5	4,5	2,1	3,2	6,1
	150°C, 3'	25,6	3,8	1,8	1,7	6,8
	160°C, 3'	24,6	3,6	1,8	1,2	6,8
	170°C, 3'	23,9	3,2	1,9	1,0	7,5
Zostera	untreated	38,6	5,6	1,4	2,9	6,9
	untreated, 2%	39,9	5,9	1,3	0,5	6,8
	160°C,1'	39,7	5,8	1,7	1,4	6,9
	160°C,1', 2%	39,6	5,8	1,7	1,2	6,8
	160°C,5'	37,8	5,5	1,4	1,4	6,9
	160°C,5', 2%	39,9	5,7	1,8	1,6	7,0
	170°C,3', 1%	42,0	6,2	1,4	1,7	6,8
	170°C,3', 1%	41,3	6,1	1,6	1,3	6,7
	180°C,1'	39,6	5,8	1,2	1,6	6,9
	180°C,1', 2%	42,7	5,9	1,7	1,5	7,2
	180°C,5'	41,7	5,6	1,7	1,3	7,5
	180°C,5', 2%	41,8	5,6	1,7	1,3	7,5

3.3 Sugars in the soluble material (SM)

In the Table 4 is reported the sugar content of the aqueous solution after hydrolysis by sulfuric acid 1%, 1 bar (120 °C).

Table 4 – Water soluble sugars of Ulva and Zostera before and after steam explosion (from 100 g of water soluble material)

T, minutes, ox. acid		Rha	Ara	Xyl	Gal	Fuc	Glu	GalA	GluA	Total
Ulva	untreated	4,86		1,26	0,26		5,05	16,30	1,05	**28,79**
	150°C, 3'	2,30		0,52	0,15		2,05	10,42	0,54	**15,98**
	160°C, 3'	2,71		0,70	0,15		2,81	9,09	0,58	**16,06**
	170°C, 3'	2,94		0,76	0,16		3,05	9,86	0,63	**17,41**
Zostera	untreated		0,07	0,23	0,51	0,03	5,19	0,21	0,00	**6,22**
	untreated, 2%		1,18	1,05	1,11	0,04	2,85	1,01	0,15	**7,38**
	160°C,1'		0,43	0,98	1,07	0,23	5,82	0,94	0,13	**9,60**
	160°C,1', 2%		1,21	2,31	1,49	0,34	3,34	2,71	0,24	**11,63**
	160°C,5'		1,02	2,25	1,26	0,24	3,97	1,13	0,18	**10,04**
	160°C,5', 2%		1,69	3,77	1,86	0,41	2,60	5,12	0,31	**15,76**
	170°C,3', 1%		2,15	4,39	2,74	0,66	1,66	2,88	0,39	**14,87**
	170°C,3', 1%		1,77	3,77	2,24	0,71	1,89	2,92	0,34	**13,64**
	180°C,1'		1,11	2,30	1,79	0,37	2,93	1,48	0,24	**10,21**
	180°C,1', 2%		0,91	3,08	1,52	0,28	2,48	4,84	0,26	**13,36**
	180°C,5'		0,99	3,89	1,79	0,26	1,31	1,55	0,28	**10,08**
	180°C,5', 2%		1,49	4,51	1,95	0,25	1,81	2,09	0,29	**12,39**

3.3.1 Highlights

Considering the Zostera case, we note that the highest ratio sugars/SM is obtained at medium severity; probably, at higher severity, degradation involves the extractable sugars, although the SM increases (see Figure 2). As shown in Figure 4, the preimpregnation with oxalic acid increases the solubility of the sugar.

Glucose is the most abundant component at low severity, while galacturonic acid and xylose are the most abundant at high severity

In the Ulva case, the soluble sugars yield was higher than Zostera and, the non-determined material was lower. The galacturonic acid was the main carbohydrate.

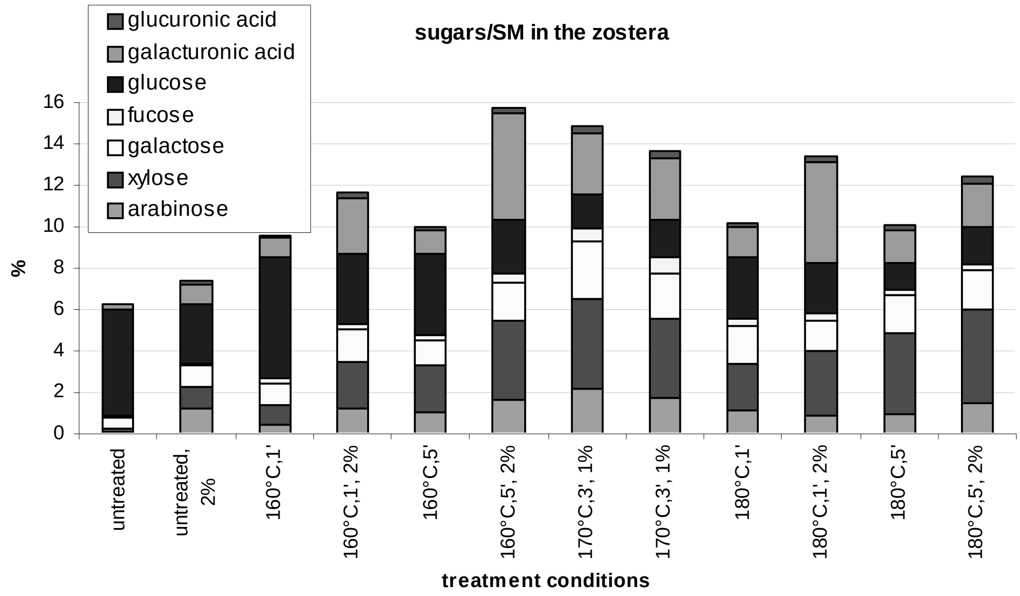


Figure 4 – Water soluble sugars obtained from steam exploded Zostera.

3.4 Sugars content of the Insoluble Material (IM)

In the Table 5 is reported the sugars content of the IM, as determined by acid hydrolysis (AH) and HPIC, in the glucose case also an alternative procedure based on enzymatic hydrolysis (EH) was examined.

As regards the data of Zostera, the large difference between the glucose produced by AH and that produced by EH (up to 3 times!) pointed out the unreliability of the AH in quantifying the sugar content in this kind of marine biomasses, in contrast to witch is largely reported for terrestrial plants.

Indeed, several different conditions to carry out the AH were tested (varying the temperature, time and H_2SO_4 concentration) the method seemed to be optimized at the highest glucose production. This evidence could point out glucose degradation during the acid is a parallel reaction to the glucan hydrolysis; as a result, the quantification is hindered. On the other hand, EH is not a degradable process, but its efficiency depends on the substrate digestibility, i.e. can be poor effective on raw material or good in pretreated materials, when the fibers are sufficiently opened.

In conclusion, the glucose content can quantified at best by using an optimised combination of SE and EH.

In Figure 5 are reported the total sugars detected in the IM at various treatment conditions.

Glucose is the main component and oxalic acid improves systematically the hydrolizability.

Table 5 – Sugars content of the water insoluble fraction of raw and steam exploded Zostera (as percent w/w).

zostera	Ara	Xyl	Gal	GalA	GluA	Glu by AH*	Glu by EH**
untreated		3,1	1,0	0,5		18,4	10,3
untreated, 2%		2,9	0,9	1,7		17,4	22,4
160°C,1'		3,1	0,9	1,1	0,2	21,1	30,1
160°C,1', 2%		2,7	0,8	2,4	0,5	21,5	36,1
160°C,5'		2,6	0,7	1,2	0,3	22,7	36,9
160°C,5', 2%		2,3	0,6	2,3	0,3	26,7	44,2
170°C,3', 1%	0,2	2,0	1,3	1,3	0,3	13,0	39,2
170°C,3', 1%	0,2	2,8	0,7	1,3	0,3	13,5	42,3
180°C,1'	0,1	2,5	1,6	1,4	0,4	17,5	36,8
180°C,1', 2%	0,2	2,4	1,6	2,5	0,4	21,0	42,4
180°C,5'	0,1	1,8	1,0	0,9	0,4	26,6	52,5
180°C,5', 2%	0,1	1,4	0,8	1,8	0,7	28,1	52,9

**AH = acid hydrolysis, **EH = enzymatic hydrolysis*

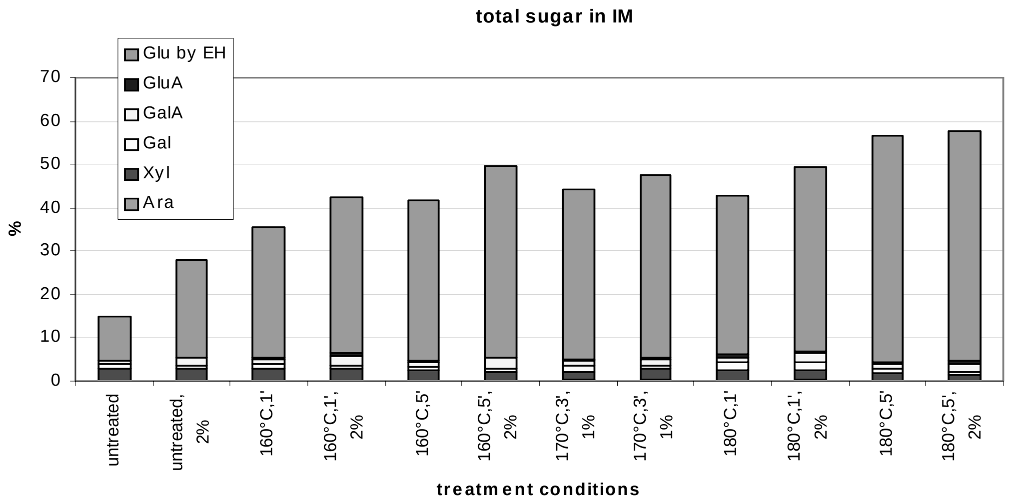


Figure 5 – Sugar content of the IM fraction of raw and SE Zostera (as % w/w).

IV: Byproducts Exploitation

Part I: Bioethanol from Zostera

1. INTRODUCTION

Bioethanol from residual biomass can provide a real contribute to substitute fossil fuels and reduce the CO_2 emissions [27, 28, 29]. Several lignocellulosic residues are examined as raw material to obtain ethanol by hydrolysing the cellulose and fermenting the produced glucose, but studies on the marine plants as source for biofuels are rarely reported in the literature [30, 31, 32, 33]. In the same time, huge amount of marine plants are deposited on the beaches of many countries every year because of the eutrophication, and usually this not-employed residue, is a calamity for the tourism [34]. Among the collectable submerged seagrasses, *Zostera marina* is one of the more diffuse: the aim of this work was to valorise this residue for the bioethanol production.

For this purpose, chemical analyses were firstly carried out to verify the availability of a sufficient amount of cellulose, the results showed a content of about 30% w/w on the DM bases of cellulose. As pretreatment, necessary for a good hydrolyzability by enzymes, steam explosion trials were carried out in the range 160-180 °C, and 60-300 seconds. The employed steam reactor was a batch plant of 1kg cycle capacity. To improve the substrate quality a pre-impregnation with oxalic acid (0-2% of the dry Zostera) were also examined. It is known in fact that the oxalate anion captures the metal ions (Ca^{++}) contained in the fibre, making the carbohydrate structure more free and hydrolysable.

The effects of the parameter variations on the soluble sugars extraction and on the cellulose hydrolyzability were studied by an experimental design and the best conditions were statistically predicted. On the substrate, deriving from the steam exploded Zostera at the best treatment conditions, tests of ethanol production were carried out at the bench scale and the yields were optimized varying the solid/liquid ratio, the enzyme and the yeast load.

2. MATERIALS AND METHODS

2.1 Carbohydrates characterization

The carbohydrate content was determined by transformation of the polymeric sugars in the monomers and detecting them by HPIC (High Performance Ionic Chromatography).

For the cellulose content determination, about 1 g of the crude material was dried and milled at 50 mesh, then put in a 100 ml flask. 20 ml of water and 100 mg (as protein content) of an enzymatic mixture, containing the enzymes Celluclast and Novozyme (Novo Nordisk) was added. The flask was kept a 50 °C under stirring for 3 days, and then the suspension was filtered to remove the solids and diluted for the glucose determination by HPIC.

For the soluble sugars determination, the material was subjected to the zosterine extraction [2], then the crude zosterine was hydrolyzed by sulphuric acid 3% solution at 120 °C, 1h and the solution was analyzed by HPIC as below.

2.2 *Impregnation with oxalic acid*

200 g of the raw Zostera (DM 90%) were put in a vessel and sprayed with 250 g of water solution containing 0, 2 or 4 g of oxalic acid. The humidified material was then manually homogenized for ten minutes, avoiding the dry leaves remaining.

2.3 *Steam explosion*

The impregnated Zostera (450 g) was loaded in a batch steam explosion plant, 1kg of treating biomass capacity. Temperature of steam was set at 160, 170, 180 °C, while time was set at 60, 180, 300 seconds, respectively for each run predicted by the experimental design. After the explosion, the product, collected in the expansion chamber, was collected and weighted. It was then sealed in plastic bag and frozen.

2.4 *Water washing*

About 100 g of exploded material was extracted two times with 300 ml of hot water (65 °C), and then filtered on paper filter WHATMAN 4 using vacuum pump. The insoluble residue was washed with 100 ml of water, filtered and the liquid phase diluted until to obtain 1 liter of aqueous extract. About 200 ml of aqueous extract was evaporated to recover the dried soluble material (SM), while 100 ml was frozen and stored. Both samples were analyzed. The insoluble material (IM) was dried overnight at 60 °C, weighted and stored in plastic bags; amounts of 5 g were grinded at 50 mesh for the chemical characterization.

2.5 *Design of the experiments*

The planning of the experiments was made by a software called “Essential Experimental Design”(‘97). It provides the empiric correlation to the process variables by interpolation equations. Investigated variables were 3: temperature, time and percentage of oxalic acid; two centre points were chosen and the “Level” was set at higher resolution (full factorial). The required runs resulted 10, as they were obtained as combination of the extremes values of the parameters and one central condition to be carried out in duplicate.
Variables were studied in the following ranges:

Temperature (°C)	*160-180;*
Time (sec)	*60-300;*
Oxalic acid (% w/w)	*0-2.*

2.6 *Separated Hydrolysis and Fermentation (SHF)*

The biomass was hydrolysed using the cellulolytic complex Celluclast 1,5L and Novozym 188 (Novo Nordisk).

5 g of dry substrate were put in a flask and 100 ml of a 50mM acetic buffer solution was added (pH 5). The mixture of the enzymes (Celluclast/Novozyme 6:1) was added as protein/substrate ratio 0.06 w/w (0.5 FPU/mg$_{\text{-protein}}$), and then the flask was kept at 50 °C for 3 days under stirring. Samples (2 ml) were picked up, centrifuged and analyzed for the glucose content by HPIC.

For the fermentation process 0.3 g of the yeast *Saccharomyces cerevisiae* (Sigma, type II) and 0.25 g of the yeast extract were added and the suspension was kept 3 days at 30 °C under stirring (100 rpm). At the end, samples (2 ml) were picked up, centrifuged and analyzed for the ethanol determination.

2.7 Simultaneous Saccharification and Fermentation (SSF)

It has been used the same procedure of the SHF, but yeast and yeast extract were added with the enzymes in the same time and the flask kept at 35 °C for four days under stirring [35].

3. RESULTS

3.1 Pretreatment optimization by DOE (Design Of Experiment)

For the Zostera conversion into ethanol, steam explosion pretreatment was assessed. The optimization of the pretreatment consisted in the determination of the best parameters adopted to have a substrate highly hydrolysable, with a parallel good recovery of soluble sugars by aqueous extraction. The range of temperature and time of the steam treatment was chosen on the basis of the experience, to avoid a too slight or too harsh severity: below 160 °C and 60” the fibre resulted uncooked; up 180 °C and 300” it results highly destroyed. The utilization of oxalic acid in the steam reactor would have the double effect of catalyze the hydrolysis reaction and capture the metal ions. As first approach and to limit high amount of chemicals, a max of 2g of oxalic acid for 100 g of substrate was employed to examine the quality improvement of the exploded material produced. After the definition of the parameters ranges, data were input to generate an experimental design, i.e. a right number of runs to obtain a statistic study of the results and to obtain correlation equations between the responses and the process parameters. Combining the extremes and considering two central conditions, ten runs were generated (Table 1).

Table 1 – Pretreatment conditions of steam explosion.

Run	Temperature (°C)	Time (sec.)	Oxalic Acid (%)
1	180	300	2
2	180	300	0
3	180	60	2
4	180	60	0
5	170	180	1
6	170	180	1
7	160	300	2
8	160	300	0
9	160	60	2
10	160	60	0

For each run about 3kg of exploded material were produced, and then water extraction and enzymatic hydrolysis were carried out. Water extraction let to obtain two fractions: the insoluble material (IM) and the soluble material containing zosterine (SM). On the SM acid hydrolysis was carried out and the resulting monomeric sugars were determined. On the IM, containing cellulose, enzymatic hydrolysis was carried out to determine the glucose content; for each sample the necessary amount of enzyme to have the max degree of hydrolyzability was tested, using three ratio of enzyme/substrate (E/S): 0.03, 0.06, 0.09. As reported in Figure 1, the ratio E/S of 0.06 was sufficient to have the max production of glucose for each steam treatment. Table 2 shows, for each run, the obtained glucose and the main extracted soluble sugars, reported as percent of the starting material weight.

Table 2 – Carbohydrates obtained in the fractioned substrate.

run	Glucose/IM (%)	Sugars/ES (%)
1	52.9	5.06
2	52.5	3.26
3	42.4	4.33
4	36.8	2.43
5	42.3	3.53
6	39.2	3.15
7	44.2	4.95
8	36.9	2.78
9	36.1	2.40
10	30.1	1.40

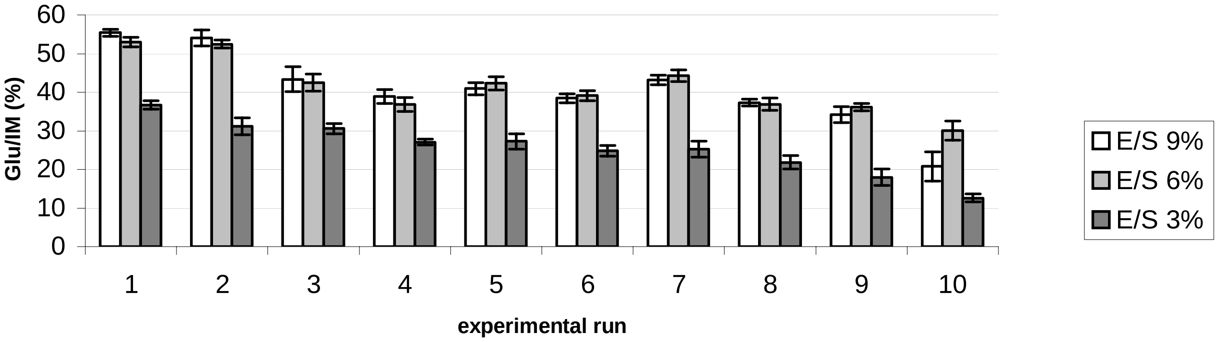


Figure 1 – Enzymatic hydrolysis of Zostera cellulose at different Enzyme/Substrate (E/S) loading.

Utilizing the experimental results of the Table 2 as input data, interpolation equations were generated:

$$\mathbf{Resp} = b0 + b1*T + b2*t + b3*ox + b4*T*t + b5*T*ox + b6*t*ox \qquad \text{[Eq. 1]}$$

Where:
Resp is the expected result (for ex: Sugar/SM);
T is the temperature of the steam reactor
t is the biomass cooking time
b0, b1, b2, bN are the coefficients;
ox is the amount of the loaded oxalic acid, expressed as % w/w of the treated dried biomass.

Table 3 shows the values of the coefficients obtained for the attended results and the correspondent correlation degree R^2.

Table 3 – Coefficients of the Equation 1.

Resp	b0	b1	b2	b3	b4	b5	b6	R^2
Glucose/IM (%)	-28.27	0.35	-0.15	18.66	-	-0.09	-	0.977
Sugars/ES(%)	-12.33	0.08	0.05	-0.47	-	0.01	-	0.978

The equation 1 is graphically reported for the two indicate responses; because it is impossible to represent a 4D graph [*y=f(T, t, ox)*], two cases have been analyzed: with oxalic acid 2% and without oxalic acid. In the case of the carbohydrates recovery in the aqueous extract (Figure 2), it is evident that the pre-impregnation of the oxalic acid lets to recover more sugars, enhancing it from 3-4 to 5-6%. Without oxalic acid preimpregnation, the predominant surface is 2-3% of sugar recovery, while, with the oxalic acid, the predominant surface is 4-5% of sugar recovery.

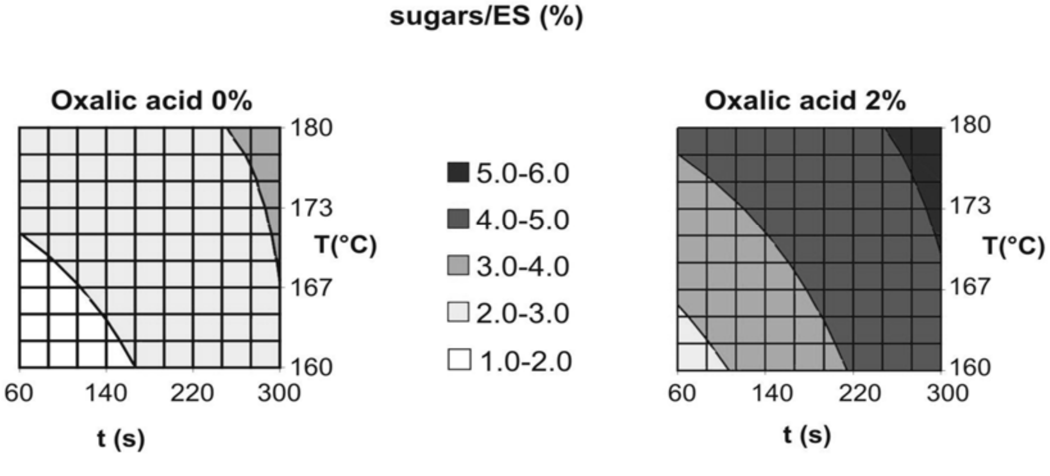


Figure 2 – Prediction of the soluble sugars recovery respect to the exploded substrate (ES), without and with oxalic acid pre-impregnation.

In the case of the glucose produced by enzymatic hydrolysis performed on the insoluble residue (Figure 3), the predicted responses have given analogue results, but the oxalic acid effect is less large than those shown in Figure 2.

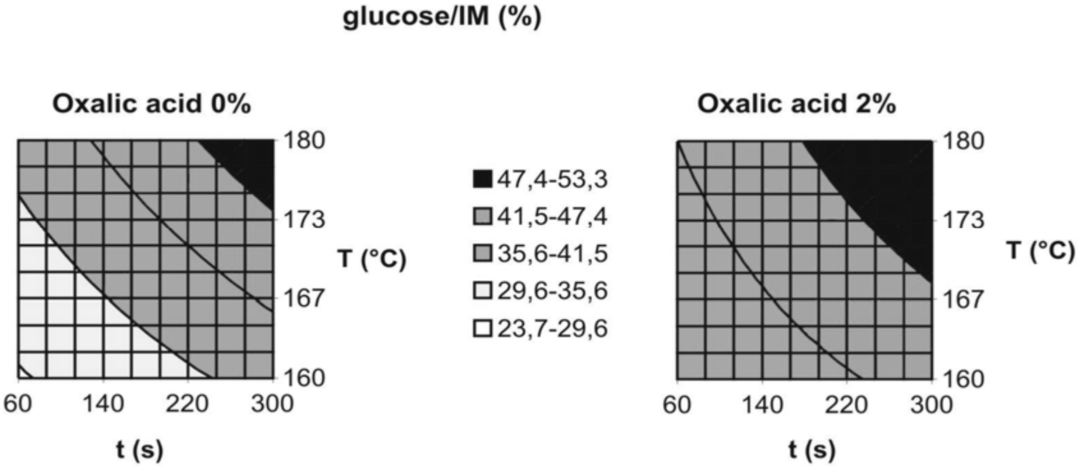


Figure 3 – Prediction of the glucose produced by enzymatic hydrolysis of the insoluble material (IM), with and without oxalic acid pre-impregnation.
To have the max glucose production, using 2% of oxalic acid temperature can be reduced of about 7 °C or time can be reduced of about 30 seconds.

On the basis of the predicted responses, for the maximum glucose production and the maximum soluble sugars recovery, the best substrate has to be produced at 180 °C, 300 s, 2% of oxalic acid.

3.2 Conversion of cellulose into ethanol

The conversion of the cellulose into ethanol was performed in shacken flasks using 5 g of substrate (IM) in 100 ml of buffer solution (acetic acid/sodium acetate 50 mM, pH 5). The applied procedure was a SHF (separate saccharification and fermentation). All the ten substrates were tested for the ethanol production to verify the influence of the pretreatment on the fermentation. After 72 h of fermentation, samples were withdrawn and analysed for the ethanol content. Results have shown that fermentation doesn't depend from the pre-treatment conditions and ethanol amount is directly correlated to the glucose production. Yields of glucose conversion into ethanol were on average of 90%. In the case of the IM, steam treated at 180 °C, 300", 2% of oxalic acid, the produced ethanol was 243 g per kg of substrate (IM) and the obtained fermentation broth was 1.5° of alcohol. On the untreated Zostera was possible to obtain only 40 g of ethanol per kg of substrate, while using the not washed steam exploded substrate the ethanol produced was maximum 144 g/kg.

3.3 Obtainment of concentrate broth

Different trials were carried out to concentrate the ethanol in the fermentation broth. For this purpose, increasing amount of substrate was loaded in the fermentation medium. Using not washed exploded Zostera, it was impossible to have broth with ethanol concentration higher than 1.7%, because of the increase of inhibitors loading (aldehydes deriving from steam treatment) [36-37]. In the case of the washed exploded (IM), the inhibitors were removed by water extraction, avoiding this problem. The saccharification yield slightly decreased with the increasing of the solid to liquid ratio, but the fermentation process resulted less productive at high solid/liquid ratio. To have a fermentation yield of the 70% it was necessary a four-fold increase of the yeast loading. In this case the ethanol concentration in the broth reached the 4.7°. Table 4 summarizes the results obtained at the end of the process (3-4 days).

Table 4 – Yields of fermentation at increasing solid to liquid ratio.

		IM in 100ml (g)	Yeast (g/l)	Glucose fermented/ Glucose fermentable (%)	Ethanol concentration in the final broth (v/v)
Shacken Flask 200 ml, SHF		5	3	100	1.7°
		10	3	63	2.1°
		15	3	50	2.5°
		20	3	43	2.9°
		20	6	59	3.9°
		20	12	62	4.1°
Bioreactor 2 l (anchor impeller)	SHF	20	12	70	4.7°
	SSF	20	12	67	4.5°

Other conditions: enzymes 60 mg of protein per g of substrate, hydrolysis temperature 50 °C, fermentation temperature 30 °C. In the SSF temperature was 35 °C.

Part II: Biogas from Ulva and Zostera

1. INTRODUCTION

Anaerobic digestion is a complex biological process carried out by a consortium of several different microorganisms, by means of them, in absence of O_2, organic matter is converted in biogas, constituted mainly by methane and carbon dioxide. The remaining gases are usually small amounts of molecular H_2, N_2, H_2S.
The amount of biogas produced varies with the amount of organic waste fed to the digester and temperature influences the rate of decomposition, a typical biogas composition is reported in Table 1.
The biomass transformation occurs in reactors, called *digesters*. A conventional digester is mixed, fed once or more per day, heated to a temperature of 35 °C and worked at a hydraulic retention time of 20-30 days. Under these conditions, about 60% of reduction in organic matter is achieved corresponding to a methane yield of 0.24 m^3/KgVS added. The gas produced can be used directly or processed to remove CO_2 and H_2S. Solid residue can be used, after aerobic treatment, as a compost and liquid effluent to fertile irrigation.

Table 1.- Typical Biogas Composition.

Methane	50-80%
Carbon dioxide	35-40%
Hydrogen	1-3%
Oxygen	0.1-1%
Carbon monoxide	0-0.1%
Nitrogen	0.5-3%
Hydrogen sulphide, ammonia	1-5%
Water vapour	variable

Several types of biomass can be evaluated as potential sources for methane production (Figure 1).

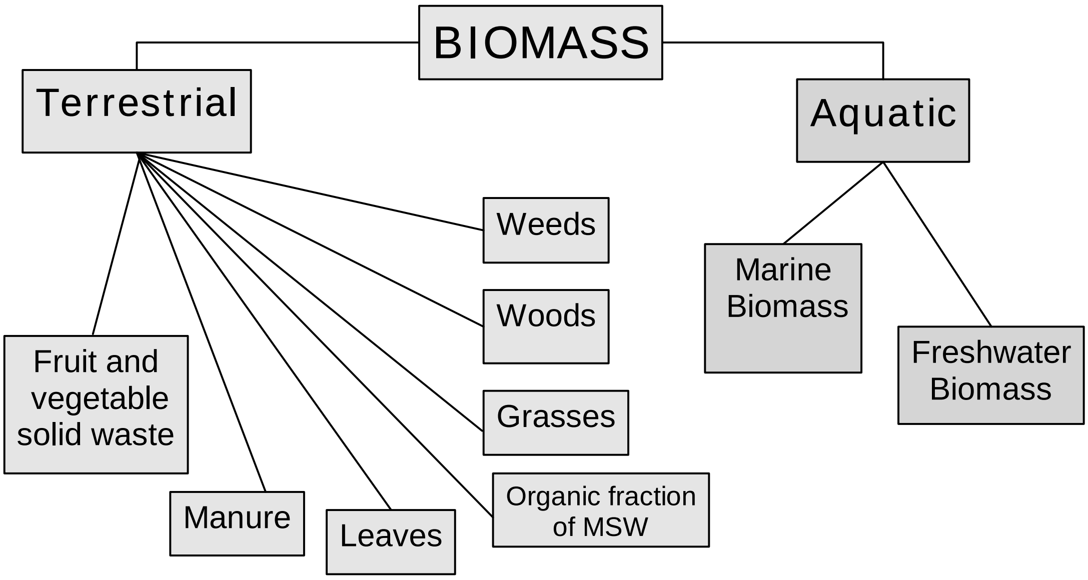


Figure 1 - Potential sources for methane production [38].

The process of biological anaerobic digestion occurs in a sequence of steps involving different types of bacteria: hydrolytic, fermentative, acidogenic and methanogenic, as illustrated in Figure 2.

The hydrolytic bacteria use cellulose enzymes to depolymerize cellulose to simple sugars. The fermentative bacteria convert these monomers to organic acids (propionic and acetic acids). The acidogenic bacteria convert these acids to hydrogen, carbon dioxide and acetate. Methanogens bacteria use these methabolites to produce methane and carbon dioxide.

The Figure 3 shows the pathway of the main constituent's decomposition until the biogas production.

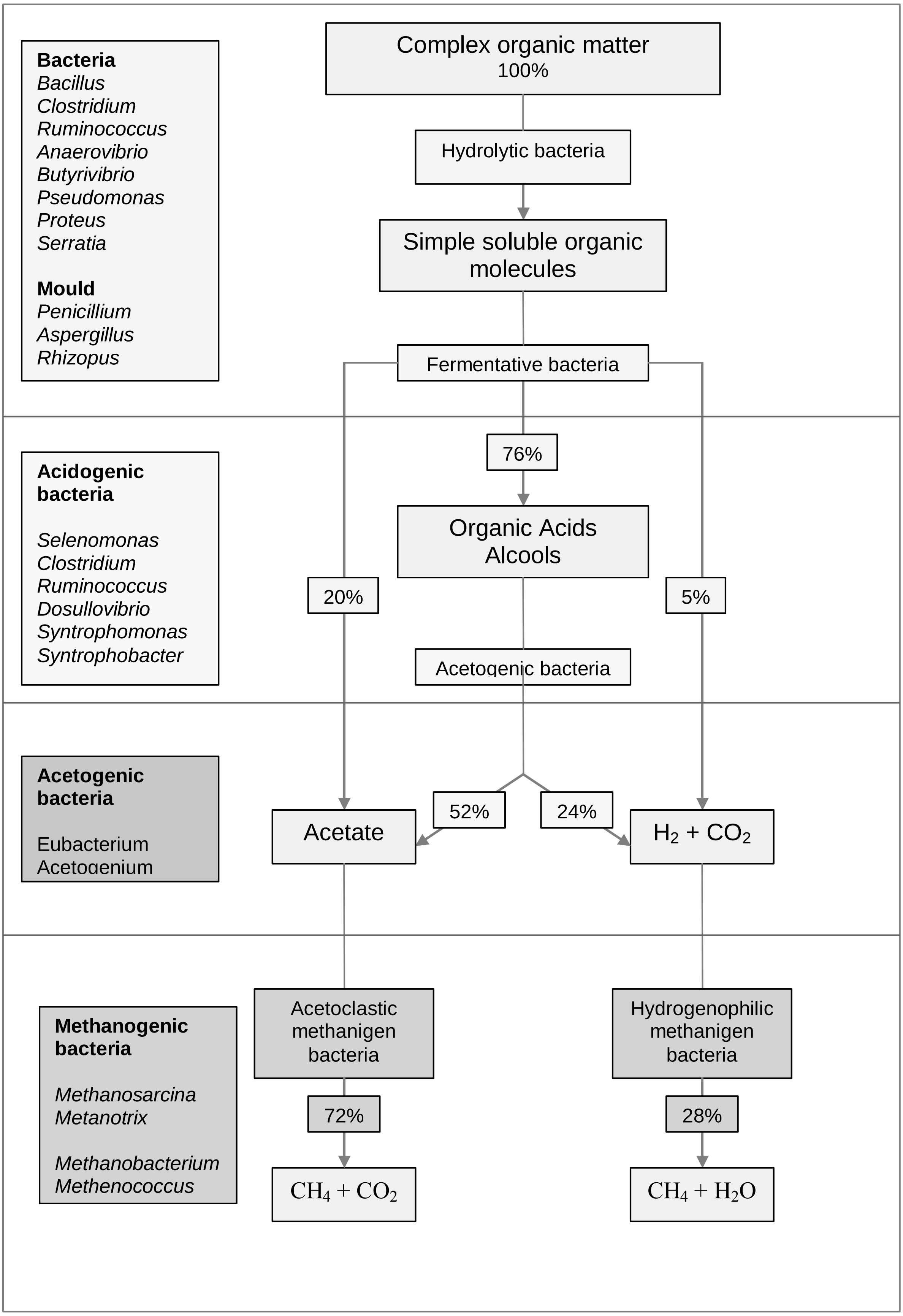


Figure 2 - Process scheme of anaerobic digestion.

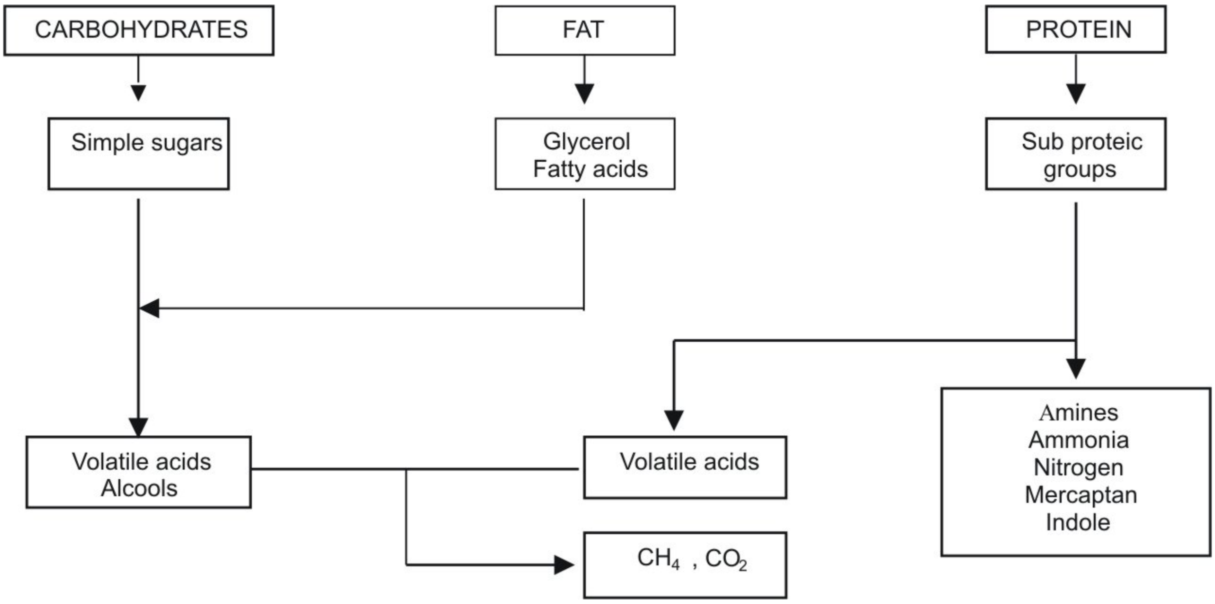


Figure 3 - Anaerobic decomposition plan of organic matters.

Anaerobic processes can be divided into:

1. Waste type treated and solid content;
2. Thermal conditions (mesophilic or thermophilic);
3. Process modality (batch or continuous).

A comparison between various parameters of anaerobic digestion process is shown in Table 2, while in Table 3 are reported the references about methane yields from different biomass.

Table 2 - Comparison between different parameters of anaerobic digestion process.

Digestion process	Description	Advantages	Disadvantages
Dry	Dry solids contents > 25-30%	Compact, lower energy input, better biogas quality (<80% CH_4), maintenance friendly	Restricted mixing possibilities
Wet	Dry solids contents < 15%	Better mixing possibilities	Higher energy input, lager reactor
Mesophilic	Digestion temperature between 25°C and 35°C	Longer process time (14-30 days), slower rate	Low energy input
Thermophilic	Digestion temperature between 50°C and 70°C	Shorter process time (14-16 days), higher degradation, faster rate	Higher energy input
Batch	Substrate in closed reactor during whole degradation period		Unstable biogas production
Continuous	Reactor is filled continuously with fresh material	Constant biomass production through continuous feeding	

Table 3 - Literature data of yields of methane from several biomass.

Biomass	m^3/KgVS	References
Sorghum	0.26-0.39	[39]
Wheat straw (NaOH treated)	0.38	[40]
Corn stover	0.36	[41]
Kelp (*Macrocystis)*	0.39-0.41	[39]
Laminaria	0.26-0.28	[39]
Sargassum	0.26-0.38	[39]
Ulva	0.20	[42]
Ulva	0.22-0.33	[43]
Napiergrass	0.19-0.34	[39]
Poplar wood	0.23-0.32	[39, 44]
Plane tree	0.32	[45, 46]
Vine shoot	0.31	[47, 44]
Willow	0.13-0.30	[39]
Water hyacinth	0.19.-0.32	[39]
Sugar cane	0.23-0.30	[39]
MSW	0.20-0.22	[39]
Avicel Cellulose	0.37	[39]

2. MATERIALS AND METHODS

Ulva and Zostera, submitted to steam explosion treatment (Ulva: T=170 °C, t=180’; Zostera: T=180 °C, t=90’), then dried and ground, were used in anaerobic digestion tests (Figure 4).

1-liter bottles were used as digesters. The digesters were kept in a room at temperature of 35-36°C. In each bottle were added 50 g of biomass and 900 ml of inoculum taken from Tecnoparco water treatment plant (Pisticci, Italy). Each test was carried out in triplicate. The digesters were run in a batch-mode, and were manually mixed for about 15 seconds everyday. Tests were stopped when gas production terminated.

Total solids (TS): The samples were dried at 105 °C for 24 h and then weighed.

Volatile Solids (VS): Total Solids were slowly burnt in an oven at 600 °C for 8 h and the resulting ash weighed. VS were measured as the difference between TS and ash.

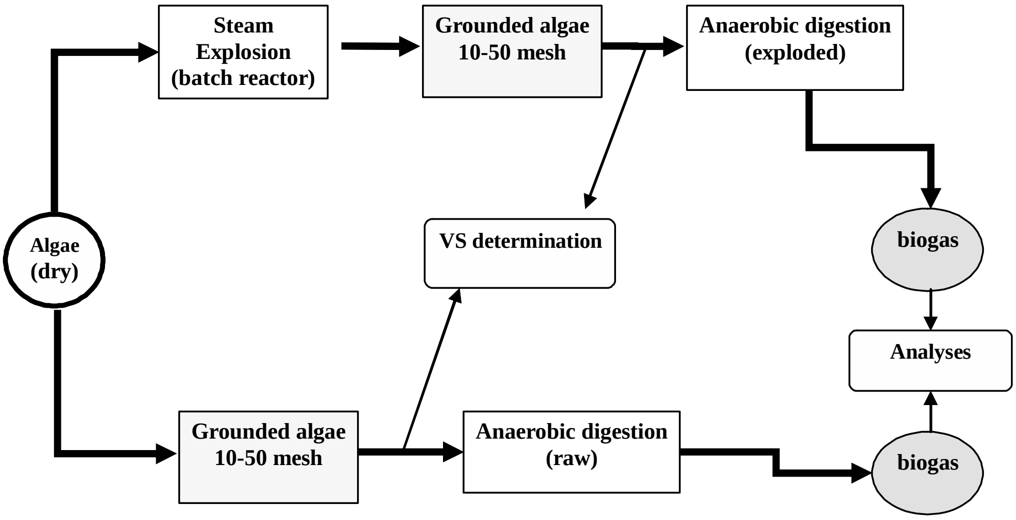


Figure 4 - Scheme of the experimental plan.

Biogas composition: Biogas was sampled as shown in the Figure 5 and the analysis was performed with a GC VARIAN. The gas mixture was eluted on a Carboxen 1000(HP) packed column with Helium as carrier gas and detected by a TCD detector. The column oven program was:

1. 5 min at 35 °C
2. From 35°C to 225 °C with a heating rate of 30 °C/min
3. 2 min at 225 °C

The determination of percentage of CO_2, N_2, O_2 and CH_4 is performed with an external calibration obtained with two standards gas certified mixtures. Biogas samples were analysed daily for the methane content. As example, chromatograms of standard and sample are reported in the Figure 6.

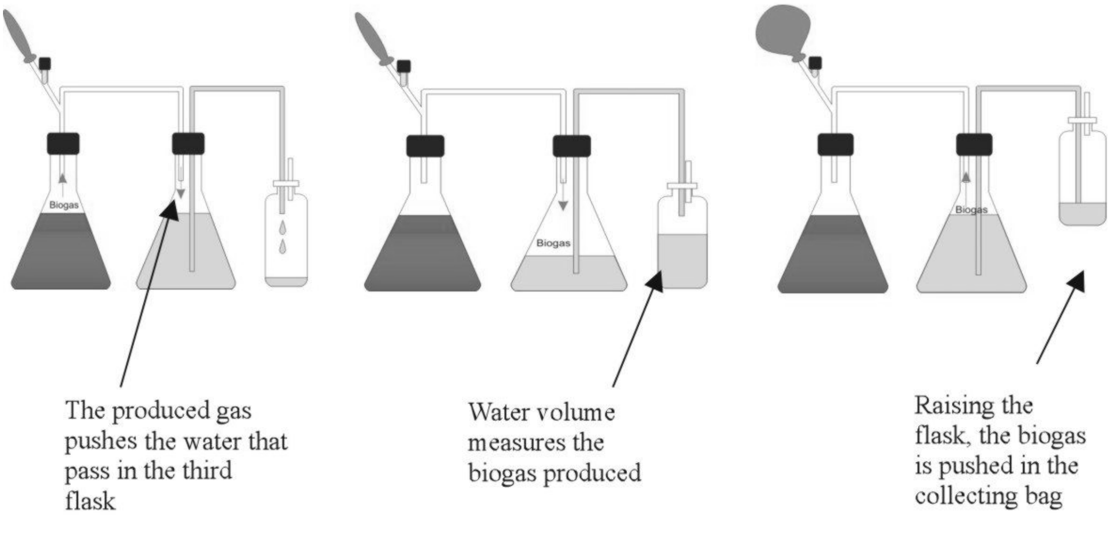
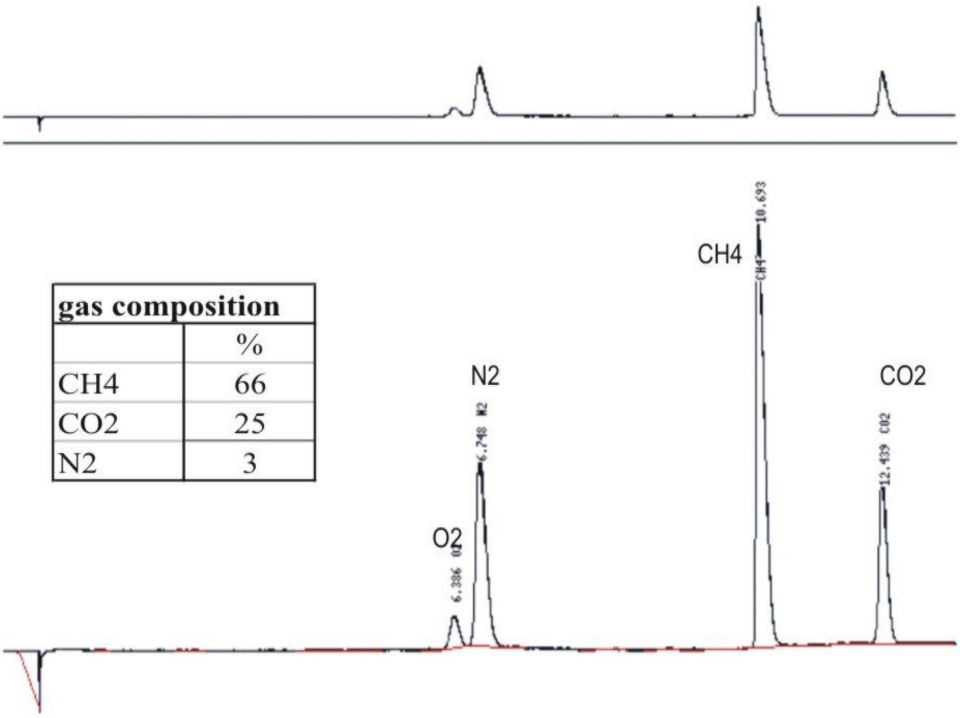


Figure 5 - Sampling procedure.



gas composition	
	%
CH4	66
CO2	25
N2	3

Figure 6 - Chromatograms (GC) of standard (up) and biogas (down) obtained from exploded Ulva.

3. RESULTS

3.1 Ulva armoricana methanisation

As shown in Figure 7 the daily productivity is averagely higher in exploded material than in raw. This is probably due to defiberization effect of steam explosion treatment.

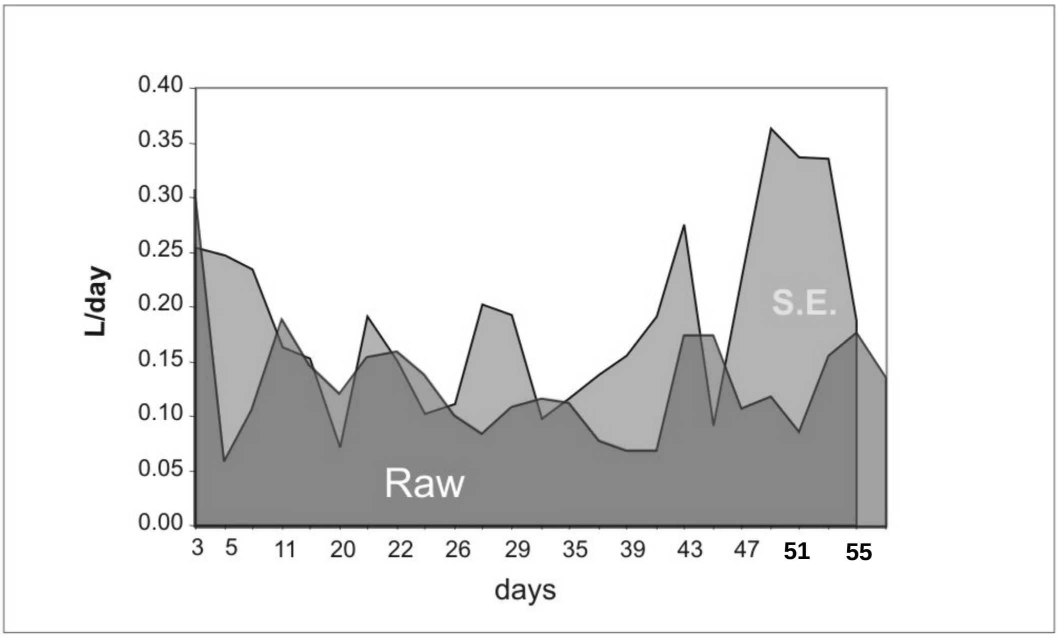


Figure 7 - Daily biogas productivity from *Ulva armoricana* raw and steam exploded.

To compare the biogas and methane yield between raw and exploded Ulva, we have represented data in terms of m^3/kg VS.

Concerning the biogas, SE material yield is 15% higher than raw (Figure 8) ; the difference increases considering the methane yield: 37% (Figure 9).

This difference is due to the better methanisation that occurs in exploded material as pointed out in Figure10, where relative percentage of methane is reported.

In the exploded material there are more free acids derived from SE pre-treatment, that are rapidly converted into methane; moreover there are better conditions for the acidogenic bacteria work, because of the prehydrolysed material presence, coming from the SE pretreatment.

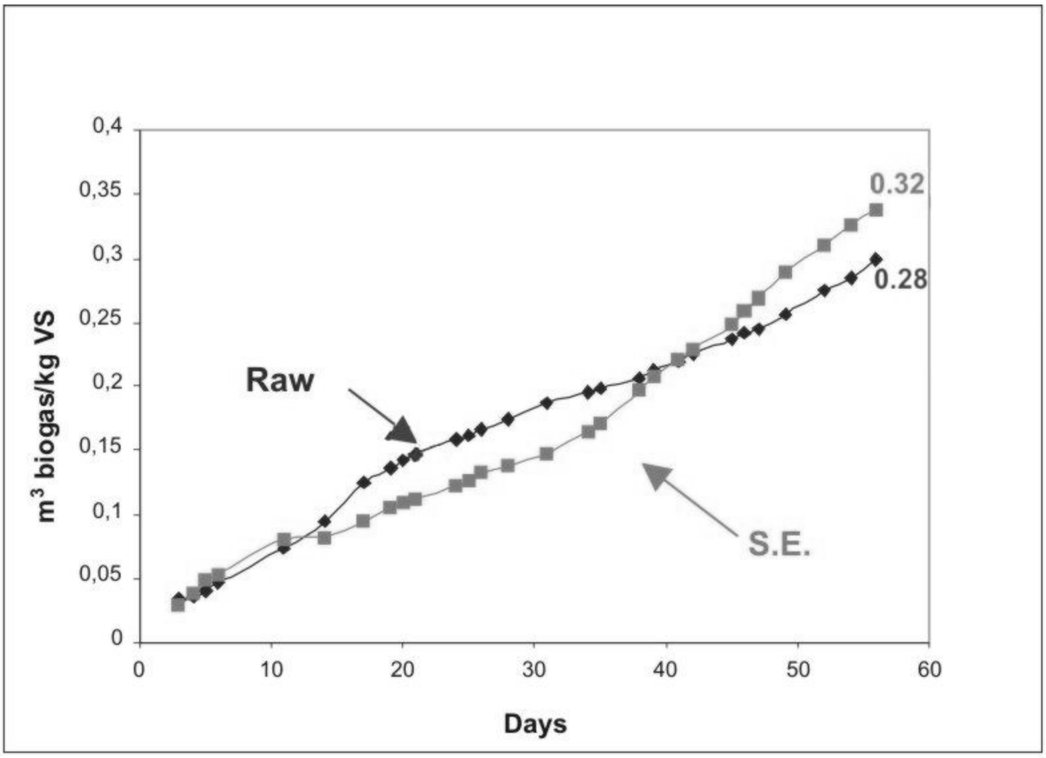


Figure 8 - Biogas production from *Ulva armoricana* raw and steam exploded.

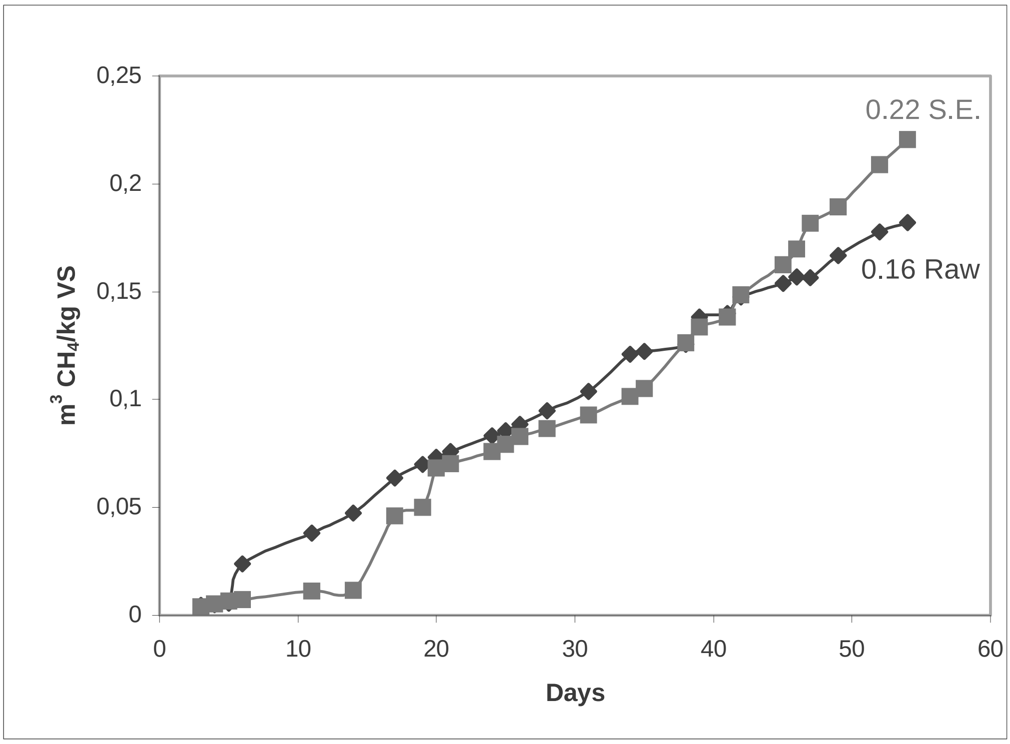


Figure 9: Methane production from *Ulva armoricana* raw and steam exploded.

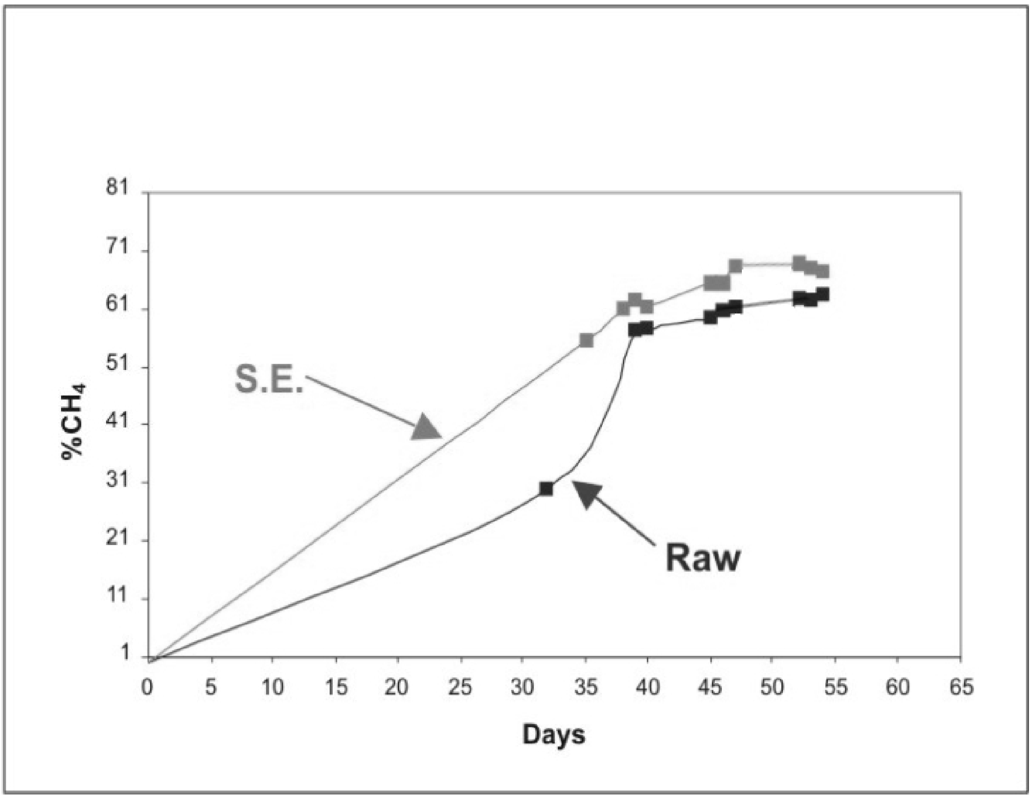


Figure 10: CH_4 percentage in biogas from *Ulva armoricana* raw and steam exploded.

3.2 *Zostera marina methanisation*

Zostera data have pointed out a greater S.E. effect in term of major biogas productivity (Figure 11), biogas and methane yield (Figures 12 and 13) and methane percentage (Figure 14). Methane yield of S.E. material is even triple respect to raw material.

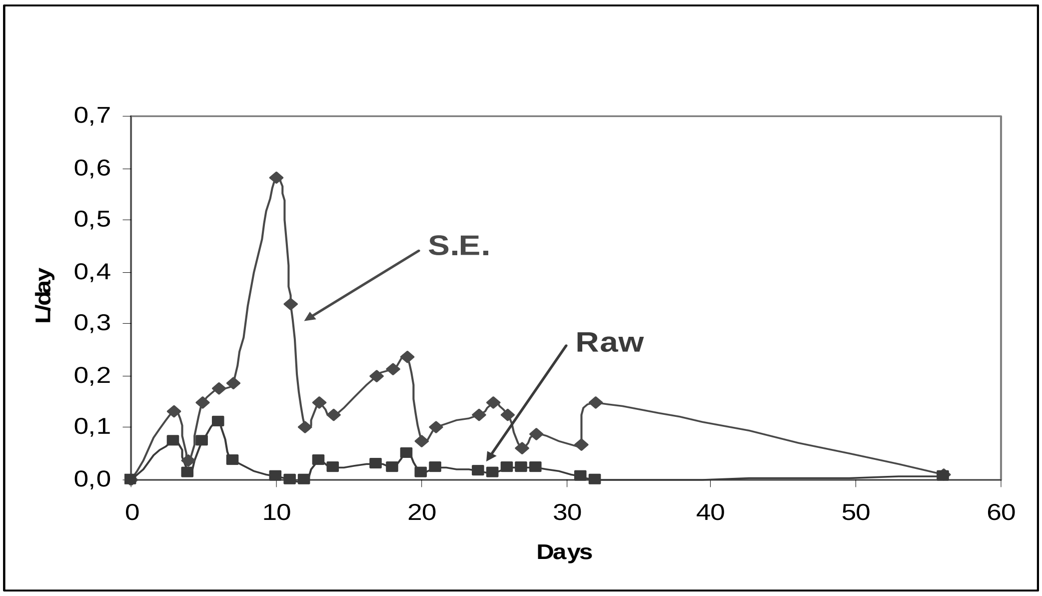


Figure 11 - Average biogas productivity from *Zostera marina* raw and steam exploded.

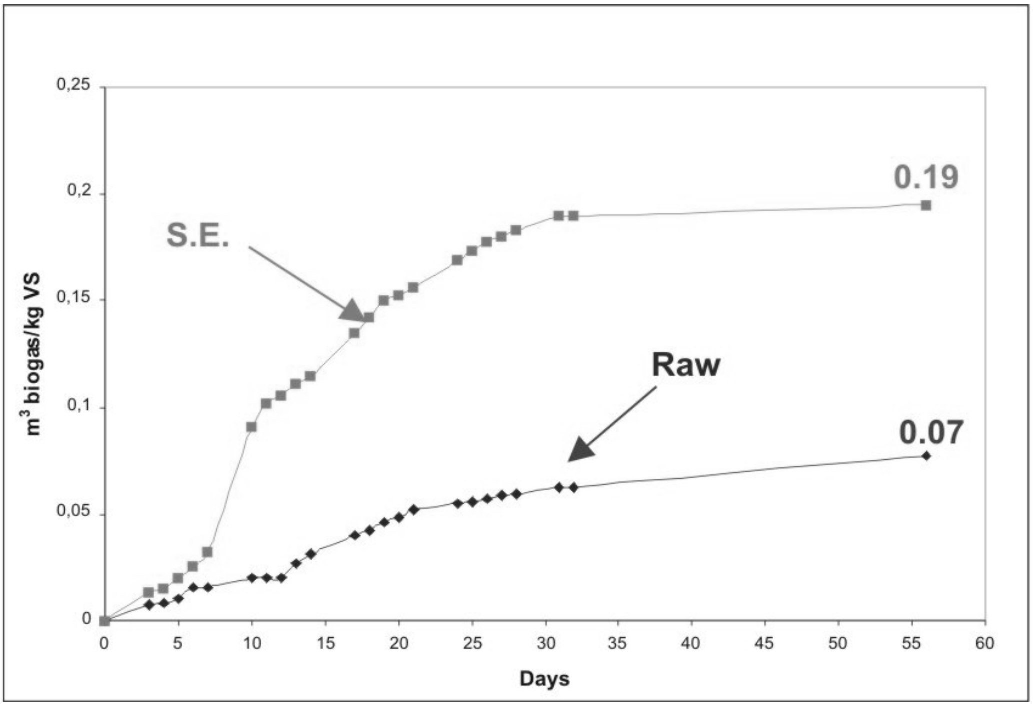


Figure 12 - Biogas production from *Zostera marina* raw and steam exploded.

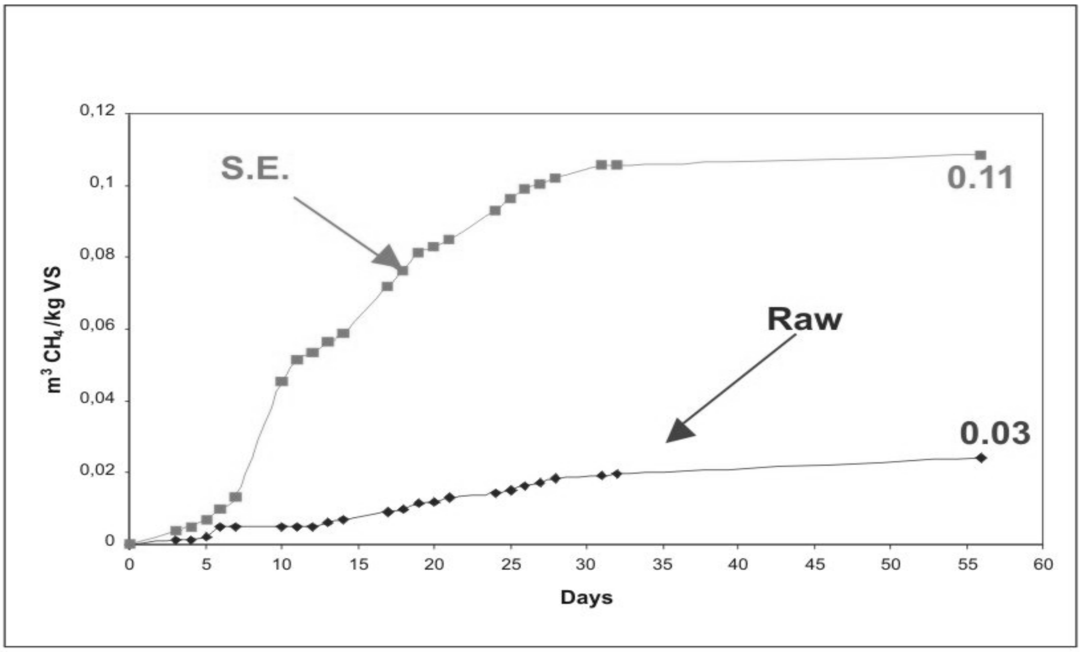


Figure 13 - Methane production from *Zostera marina* raw and steam exploded.

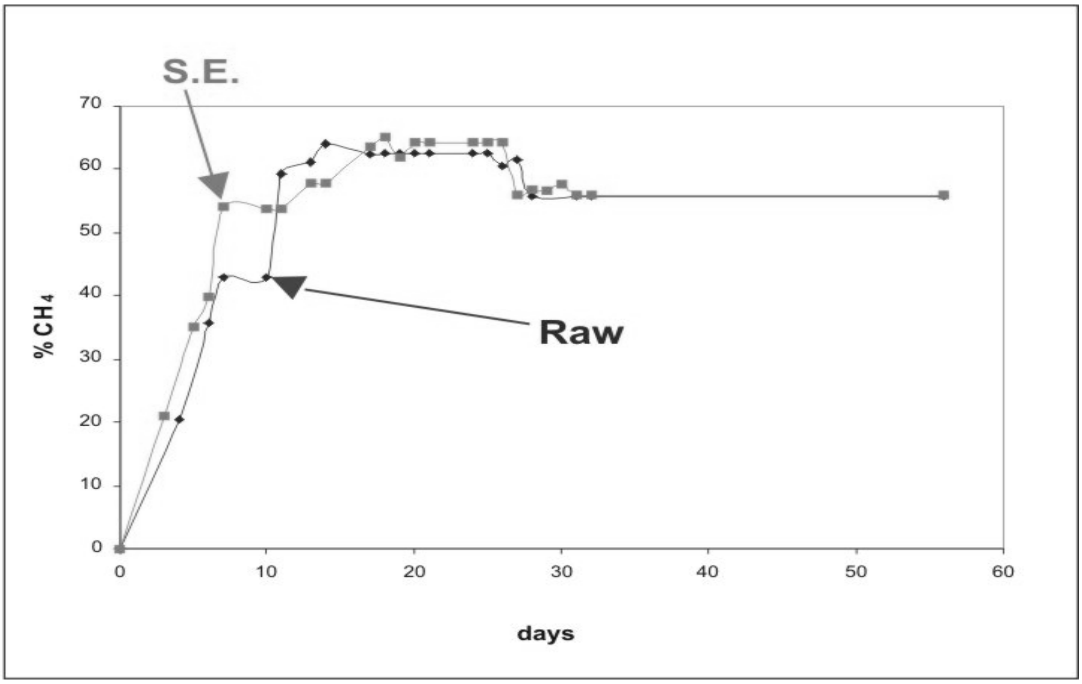


Figure 14 - CH_4 percentage in biogas from *Zostera marina* raw and steam exploded.

The comparison between Ulva e Zostera (Figure 15) point out a more favourable utilisation of Ulva to produce methane, in the both case of crude and exploded material.
Table 4 shows a summary of the final results obtained studying the biogas production from Ulva and Zostera.

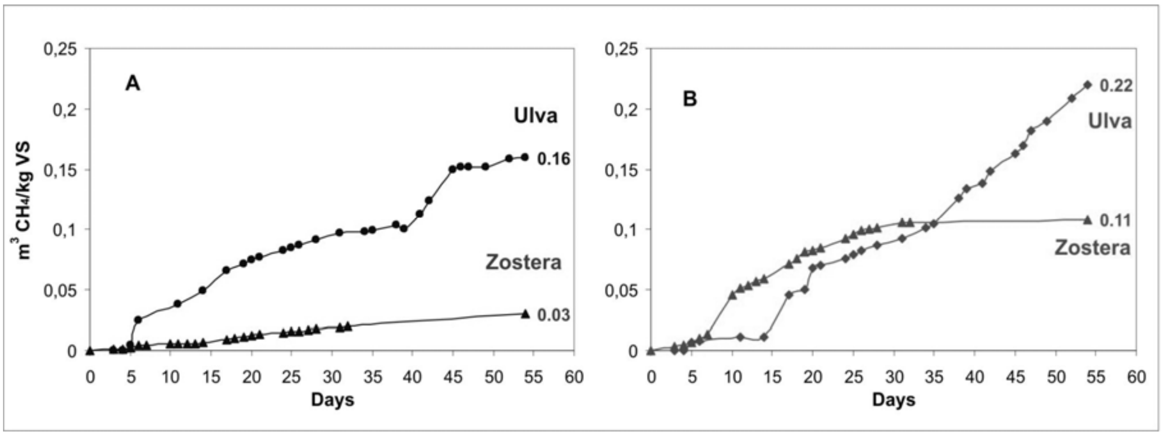


Figure 15 - Comparison between raw (A) and exploded (B) methane yield.

Table 4 - Influence of steam explosion on algae and marine plant methanisation in batch digester.

	Unit	Ulva raw	Ulva S.E.	Zostera raw	Zostera S.E.
Pretreatment (S.E.)	-	-	170° - 180’’	-	180° - 90’’
biomass	g	50	50	50	50
Volatile solids	%	54.96	53.47	53	59
Experimental time	d	54	54	54	54
Produced gas	L/g	0.16	0.20	0.05	0.12
Methane content	%	63	67	62	64
Methane Yield	m^3/kgVS	0.16	0.22	0.03	0.11

V: Techno-Economic Analysis of Algae Pre-Treatment

1. INTRODUCTION

Among the numerous processes of biomass treatment, Steam Explosion (SE) has been object of great interest from 80s, when steam exploded lignocellulosic materials were considered as polysaccharides source for ethanol production [48]. Moreover, the debate about the best pretreatment system is still open and the continuous development of new methods can be pointed out. After many publications about the technical and economical viability, it has been set up of a SE based mill producing pulp paper from straw, in Oregon and announced the implementation of an existing ethanol plant in Babilafuente (Spain) to use straw as feedstock. It is argued that if SE technology spreads in these fields, the equipment would be cheaper because of the scale economy and competition; as well the system's performances would be improved, however, there is a lack of information about the needs breakdowns, in terms of energy and chemicals. These data are basic requirement of scaling up simulations and their experimental attainment has been the object of the first part of this work.

Costs evaluation of the steam explosion treatment employed on the Zostera and Ulva is born because this process became useful as:

- SE improves the substrate quality because the biogas production increases of 37% in the case of the treated Ulva and doubles in the case of treated Zostera;
- SE let a Zostera cellulose conversion into ethanol with over 90% yield (without SE the yield was max 38%);
- SE increases the sugar extractability using hot water: until ten time in the case of the Zostera and of the 20% in the case of Ulva.

The increment of the products obtained after the SE treatment represents an income that has to be measured to compare it with the treatment costs. In this work this income was not evaluated because syrups of sugars (oligomers from biomasses), bioethanol and biogas haven't yet a well defined market. The purpose of this study was then limited to estimate the SE treatment cost for the two new investigated biomasses: *Zostera marina* and *Ulva armoricana*.

2. METHODS

2.1 The steam explosion treatment

A preliminary experimentation on a batch steam explosion plant (1 kg of biomass per cycle) was carried out to determinate the best treatment condition for the Zostera and Ulva at pilot scale. The trials on the batch plant were consisted in the exploring a range of time and temperature to have the best treatment conditions for a good quality product. The product quality of the exploded Zostera and Ulva was determined as sugar extractability, ethanol production, biogas production rates. Data obtained on the batch scale were used for the process simulation and the costs determination at industrial scale (continuous plant).

At the ENEA Research Center of Trisaia (South Italy) is operating since about 13 years a continuous SE pilot plant capable of treating up 350 kg/h of biomass, at 50% humidity. Beside the SE machinery, it is operating a fractionation section by which the biomass can be fractioned by aqueous extractions. This section is constituted by thermostated slurry thanks of 4 m^3 and multistage belt filters, typical of the pulp paper industry. This plant has been equipped with devices to measure the consumption of electricity, steam, water and chemicals.

The Figure 1 shows the continuous steam explosion reactor, the Figure 2 shows the scheme of the plant with the extraction sections and the Figure 3 shows a view of the ENEA plant.

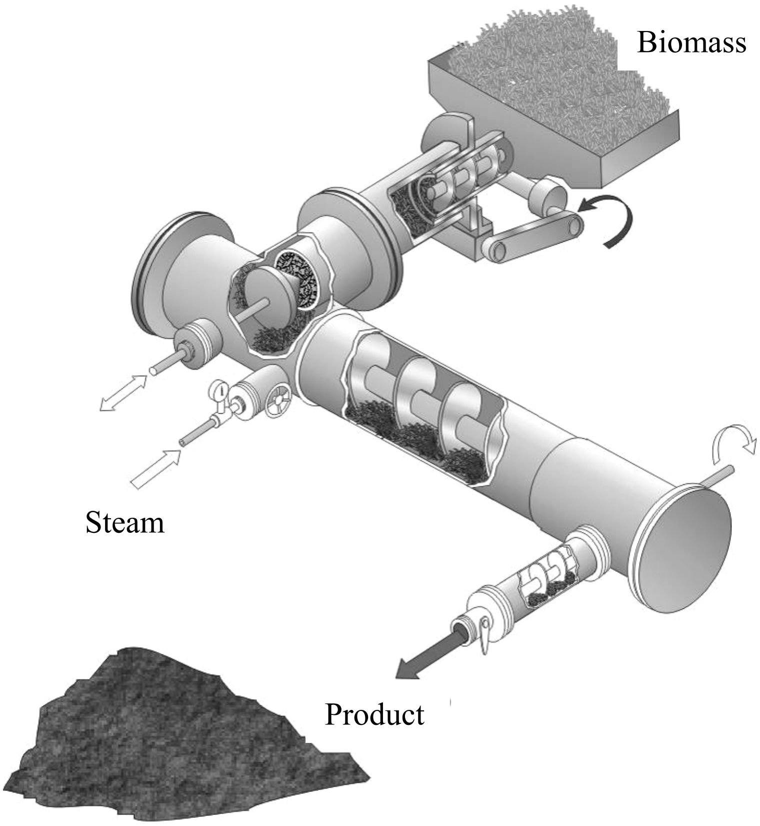


Figure 1 – Steam explosion continuous reactor.

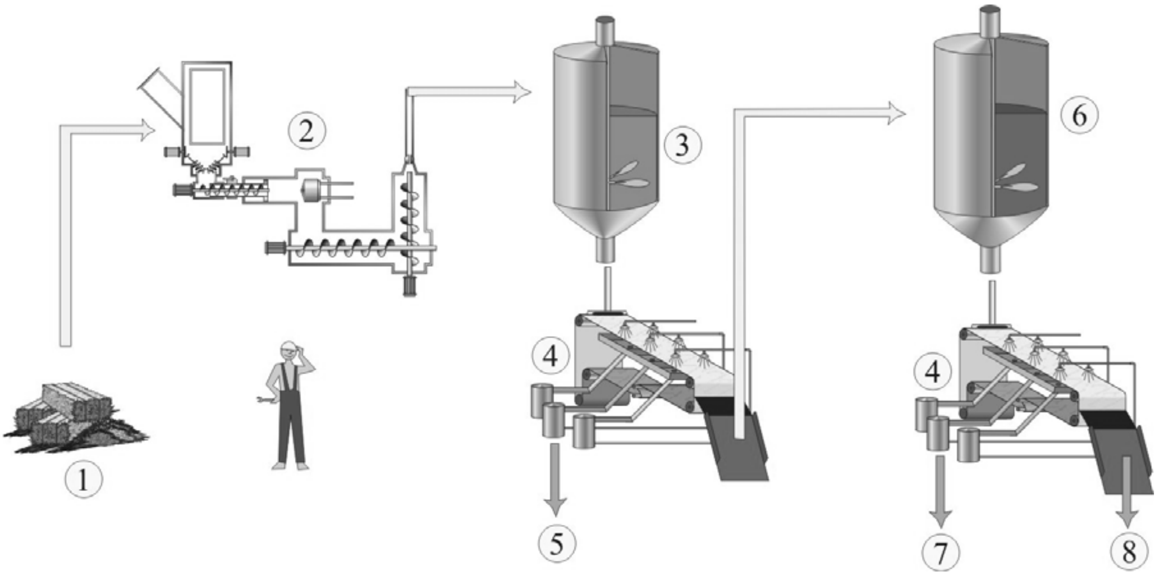


Figure 2 – Scheme of steam explosion plant with extraction section: the biomass (1) is continuously steamed and exploded in the digester (2), then slurried with warm water (3) and filtered with a belt machinery (4) to recover hemicellulose (5). The residue is slurried with alcaline solution (6), then filtered to separate the lignin (7) from cellulose (8).

Figure 3 – View of the steam explosion ENEA plant.

2.2 Cost evaluation

The specific cost of the biomass treatment has been obtained for two cases:
Case I) production of just steam exploded biomass;
Case II) production of aqueous carbohydrates solution and insoluble solid residue.

Data were collected at the steady condition, generally after 1 hour from the start up.
The measurement of electricity consumption in the Case I has been carried out by considering all the machinery for the SE process: straw chopper and all the electrical engines for the biomass handling (Archimedes screws, belts, pistons etc). Flow counters have measured the steam and water consumption. In the Case II other five items (2 stirrer, 4 pumps, and 1 belt filter) have been considered.
The hot just exploded biomass is sufficient to keep the temperature of the soluble carbohydrates extraction tank around the optimal process temperature of 60 °C. The cooling water for the biomass feeder and belt machineries has been separately measured.
The collected data have been used to carry out also a techno-economic evaluation for a medium size plant able to treat 20,000 t/y (10000 t_{DM}/y) of biomass; assuming 334 working days, it corresponds to a scaling factor of 7.13.
The scaling up cost has been based on pilot plant costs and the specific consumptions data.
The purchasing cost (PC, the cost of basic machineries), has been evaluated by using the so-called Lang factors that allow estimating the cost of common items in chemical industry on the basis of the relationship [49]:
$c=c_R*s^n$
Where c_R is the reference cost; s the scaling factor; n the Lang factor.

The other direct fixed costs (DFC), included income, have been calculated as PC quota. Devaluation, maintenance, insurance and plant management (operational costs) have been evaluated as DFC quota. Devaluation has been assumed linear in time, with a plant life of 15 years and a dismissal value of 10%. Other operational costs have been referred to the Italian market.

It has been assumed that nine workers on shift and three supervisors operate the plant.

The manpower cost has been used as reference value in evaluating the cost of administration and process monitoring (chemical analysis).

The cost of steam generation has been based on the use of methane as fuel.

The consumption of water, steam and chemicals has been evaluated as proportional to the treated biomass, because they are related to the intrinsic process chemistry. Lab experiment showed the extraction yields does not depend on scale. The term of electricity consumption has been calculated assuming a direct proportionality with the treated biomass for the biomass feeding section, and an exponential factor of 0.5 for the other machineries. The techno-economic analysis stops at the production of a liquid stream and wet insoluble steam exploded residue for which data were experimental obtained [50, 51, 52, 53, 54].

3. RESULTS

3.1 Flow sheets and streams

The flow sheet of the SE treatment followed by aqueous extraction is reported in Figure 4.

For the Zostera, a medium severity treatment, suitable for a good conversion of this substrate into ethanol, was considered. Data referred to treatment conditions of 180 °C and 300 seconds were then adopted.

For the Ulva, more slight condition was adopted: 170 °C, 180 seconds, considering that higher severity resulted too destructive.

For both the biomasses, the streams are normalized to the treatment of 100 Kg_{DM}/h and reported in the Tables 1-2.

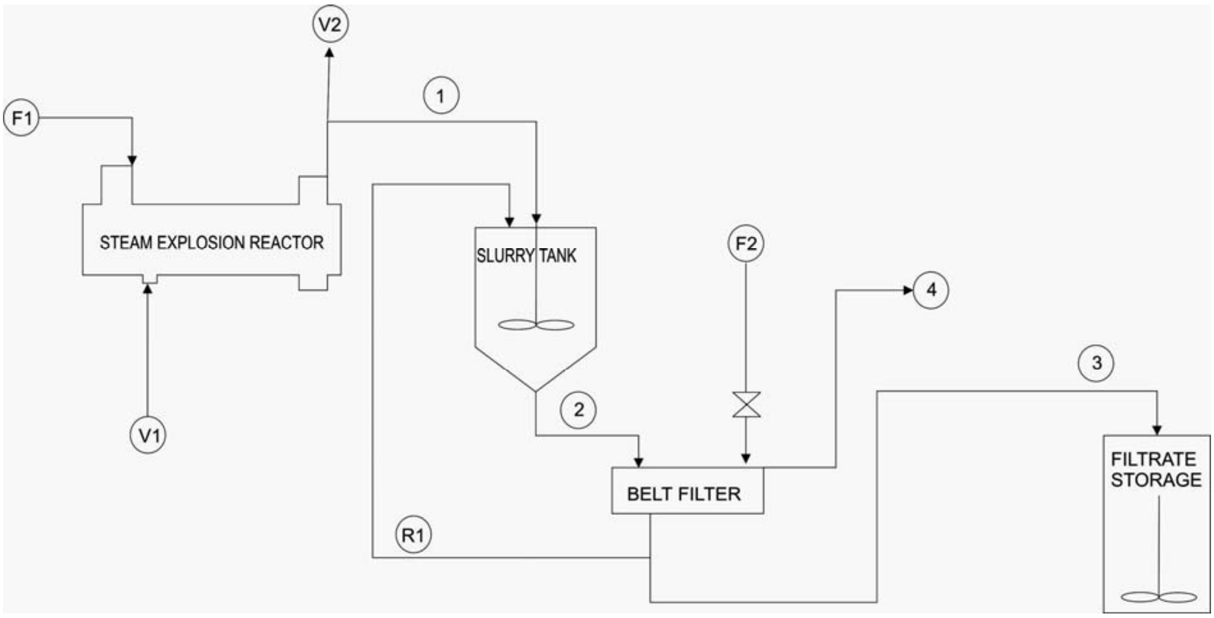


Figure 4 – Steam Explosion and aqueous extraction plant scheme. Single number means product, Fn feed, Vn vapors, Rn recycling.

Table 1 –Flow sheet for the Zostera treatment. In bold the measured values.

SPECIFICATION	V1	V2	F1	1	2	3	4	R1	F2
	Steam in	Steam out	Total feed	ES	Slurried	SM	IM	Solution recycling	Fresh water
total weight load (kg/h)	**100**	110	200	190	3332	628	222	3142	**660**
water weight load (kg/h)	**100**	105	**100**	95	3094	599,5	155,5	2999	**660**
solid weight load (kg/h)		5	**100**	95	238	28,5	66,5	142,5	
dry Mass %			**50**	**50**	7	4,5	**30**	4,5	

SM = soluble material; IM = insoluble material; ES = steam exploded substrate; SM/ES = 0.30; solution recycling (R1) = 5*SM (3).

Table 2 –Flow sheet for the Ulva treatment. In bold the measured values.

SPECIFICATION	V1	V2	F1	1	2	3	4	R1	F2
	Steam in	Steam out	Total feed	ES	Slurried	SM	IM	Solution recycling	Fresh water
total weight load (kg/h)	**100**	110	200	190	3490	660	190	3300	**660**
water weight load (kg/h)	**100**	105	**100**	95	3205	622	133	3110	**660**
solid weight load (kg/h)		5	**100**	95	285	38	57	190	
dry Mass %			**50**	**50**	8	6	**30**	6	

SM = soluble material; IM = insoluble material; ES = steam exploded substrate; SM/ES = 0.40; solution recycling (R1) = 5*SM (3).

For both the biomasses, the material lost in the vapour phase after the SE ranged from 2 to 8% and an average value of 5% was used. The main difference between Zostera and Ulva treatment consists in the highest soluble material recovery after water extraction in the case of the Ulva. In the Tables 3 and 4 are shown the flow of the detected components in the main streams.

Table 3 – Flow of the Zostera components referred at $100kg_{DM}/h$ of the starting raw material.

Component	**Raw (F1)**	**ES (1)**	**IM (4)**	**SM (3)**
Glu	36,0	35,2	34,6	0,6
Xyl	3,2	2,6	1,3	1,3
Ara	0,7	0,6	0,1	0,5
Gal	1,7	1,6	1	0,6
Fuc	0,3	0,2	0	0,2
GalA	3,7	3,6	2	1,6
GluA	0,4	0,3	0,2	0,1
Proteins	4	4	3,3	0,7
Lipids and fats	4			
Pigment	2	1,3	1,3	
Ash	17	17	9	8
n.d.	27,0	28,6	13,7	14,9
Total	**100**	**95**	**66,5**	**28,5**

ES = exploded substrate, IM = insoluble material after ES washing, SM = soluble material

Table 4 – Flow of the Ulva components referred at $100kg_{DM}/h$ of the starting raw material.

Component	**Raw (F1)**	**ES (1)**	**IM (4)**	**SM (3)**
Glu	10,0	9,9	8,8	1,1
Xyl	1,4	1,2	0,9	0,3
Rha	4,1	4,0	2,9	1,1
Gal	0,5	0,4	0,3	0,1
GalA	5	4,9	1,1	3,8
GluA	2	1,9	0,8	1,1
Proteins	4	4	3,3	0,7
Lipids and fats	4,8			
Pigment	1,4	1,3	1,3	
Ash	46	46	24	22
n.d.	20,8	21,4	13,7	7,7
Total	**100**	**95**	**57**	**38**

ES = exploded substrate, IM = insoluble material after ES washing, SM = soluble material

In the Table 5 are reported the specific consumption of steam, electricity and water as obtained by running the SE plant.

Table 5 – Biomass processing data obtained from operating the ENEA’s Steam Explosion pilot plant having a capacity of 175 kgDM/h.

	Case I	Case II
Steam for SE, kg/kg_{DM}	1.3	1.3
Electricity[a], kW/kg_{DM}	0.31	0.39
water[b], kg/kg_{DM}	1.9	8.5
Cooling water[c], kg/kg_{DM}	5.7	14.3

b) The biomass feeder alone contribute to $0.13\ kW/kg_{DM}$;
c) For raw material humidification to 50%, steam production and extraction;
d) For biomass feeder, and belt filters.

3.2 *Cost dependence by size plant*

The amount of the collectable dry alga is largely variable and, in particular regions, the max values can be reach the 1000 ton/year. In the Figure 5 is shown the volume of green alga collected in different breton municipalities (2003); considering a mass content of $20kg/m^3$, in S.Michel En Greve the amount per year was about 370 ton, while the total was about 1000 ton (source: CEVA).

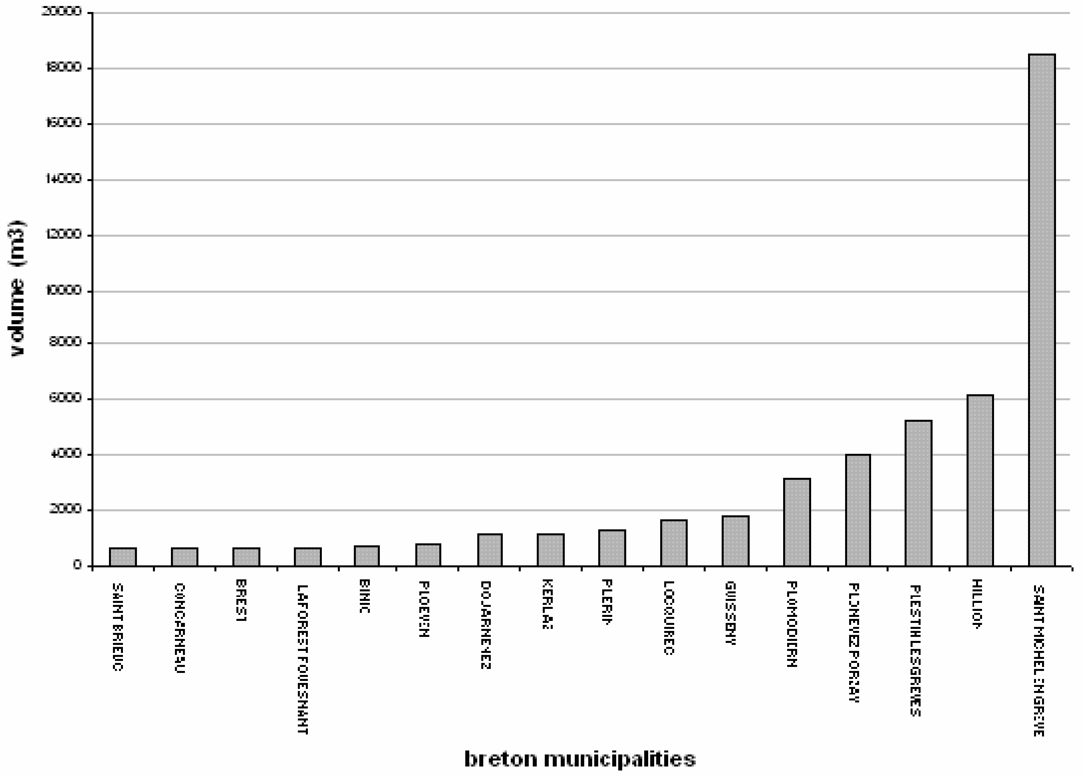


Figure 5 - Volume (in m^3) of green algae collected in different breton municipalities in 2003 (source: CEVA).

On the basis of the collectable algae amount, the economical analysis was carried out on a plant having 1000 DMton/year treating capacity. To consider the possibility of integrate the treatment whit other lignocellulosic material to produce pulp paper or cattle feed, the analysis was carried out also on the 10000 DMton/year plant capacity. Evaluation of the treatment cost as function of plant capacity was considered.

In the Tables 6-7 are reported the costs breakdown for the plants respectively of 1000 and 10000 ton/year treating capacity. In the both, two cases have been considered: steam exploded product (case I) and water washed steam exploded product (case II). In Figure 6-7 the specific cost of the SE biomass processing is reported as function of the plant scale for the both cases respectively. The data are expressed in Euros/kg and referred to the treatment costs and does not account for any selling.

Table 6 – Breakdown cost for a plant biomass pre-treatment based on the steam Explosion having capacity of 1000 t/year (DM) expressed in million Euros.

	expression	*case I*	*case II*
DIRECT FIXED COST		M€	M€
total Plant Direct Cost, TPDC			
Purchasing cost, **PC**	1PC	0.653	0.735
Installation	0.4PC	0.26	0.29
Piping	0.35PC	0.23	0.26
Instruments	0.3PC	0.20	0.22
Electricity suppling	0.15PC	0.10	0.11
Buildings	0.35PC	0.23	0.26
Place and Adaptation	0.15PC	0.10	0.11
total	**2.7PC**	**1.76**	**1.98**
total Plant Indirect Cost, TPIC			
Plant Engineering	0.15 TPDC	0.26	0.30
building Engineering	0.1 TPDC	0.18	0.20
Total	**0.25 TPDC**	**0.44**	**0.50**
Total Plant Cost TPC (TPCD+TPIC)	**3.375 PC**	**2.20**	**2.48**
Income	0.17 PC	0.11	0.12
total Direct Fixed Cost, DFC	**3.545 PC**	**2.31**	**2.60**
OPERATIONAL COST			
cost related to DFC			
Devaluation[a]	DFC(1-f)/n	0.139	0.156
Maintenance	0.02DFC	0.046	0.052
Insurance	0.005DFC	0.012	0.013
Plant Management	0.03DFC	0.069	0.078
Total	**0.115DFC**	**0.2662**	**0.299**
cost related to manpower			
Direct Manpower[b]	1MP	0.234	0.234
Benefits	0.4MP	0.094	0.094
Supervision	0.4MP	0.094	0.094
Labour equipment	0.1MP	0.023	0.023
Chemical Analysis	0.15MP	0.035	0.035
Administration	0.6MP	0.140	0.140
Total	**2.65MP**	**0.620**	**0.620**
methane[c]		0.030	0.030
Electricity[d]		0.041	0.054
water[e]		0.001	0.006
Total		**0.072**	**0.090**
Total operational cost		**0.988**	**1.040**
		€/kg$_{DM}$	**€/kg$_{DM}$**
specific cost,		**0.988**	**1.040**

a) n = years of plant activity, set as 15, f= plant value at dismissal set as 10%;
b) nine workers on shift, with individual gross wage of 2000 €/month (13 monthly salary);
c) 0.30 €/m^3; d) 0.12 €/kWh; e) 0.67 €/m^3.

Table 7 – Breakdown cost for a plant biomass pre-treatment based on the steam Explosion having capacity of 10000 t/year (DM) expressed in million Euros.

	expression	***case I***	***case II***
DIRECT FIXED COST		M€	M€
total Plant Direct Cost, TPDC			
Purchasing cost, **PC**	1PC	2.599	2.924
Installation	0.4PC	1.04	1.17
Piping	0.35PC	0.91	1.02
Instruments	0.3PC	0.78	0.88
Electricity suppling	0.15PC	0.39	0.44
Buildings	0.35PC	0.91	1.02
Place and Adaptation	0.15PC	0.39	0.44
total	**2.7PC**	**7.02**	**7.90**
total Plant Indirect Cost, TPIC			
Plant Engineering	0.15 TPDC	1.05	1.18
building Engineering	0.1 TPDC	0.70	0.79
Total	**0.25 TPDC**	**1.75**	**1.97**
Total Plant Cost TPC (TPCD+TPIC)	**3,375 PC**	**8.77**	**9.87**
Income	0.17 PC	0.44	0.50
total Direct Fixed Cost, DFC	**3.545 PC**	**9.31**	**10.37**
OPERATIONAL COST			
cost related to DFC			
Devaluation[a]	DFC(1-f)/n	0.553	0.622
Maintenance	0.02DFC	0.184	0.207
Insurance	0.005DFC	0.046	0.052
Plant Management	0.03DFC	0.276	0.311
Total	**0.115DFC**	**1.0597**	**1.192**
cost related to manpower			
Direct Manpower[b]	1MP	0.234	0.234
Benefits	0.4MP	0.094	0.094
Supervision	0.4MP	0.094	0.094
Labour equipment	0.1MP	0.023	0.023
Chemical Analysis	0.15MP	0.035	0.035
Administration	0.6MP	0.140	0.140
Total	**2.65MP**	**0.620**	**0.620**
methane[c]		0.300	0.300
Electricity[d]		0.237	0.277
water[e]		0.013	0.057
Total		**0.550**	**0.634**
Total operational cost		**2.529**	**2.747**
		€/kg$_{DM}$	**€/kg$_{DM}$**
specific cost,		**0.253**	**0.275**

d) n= years of plant activity, set as 15, f= plant value at dismissal set as 10%;
e) nine workers on shift, with individual gross wage of 2000 €/month (13 monthly salary);
f) 0.30 €/m^3; d) 0.12 €/kWh; e) 0.67 €/m^3.

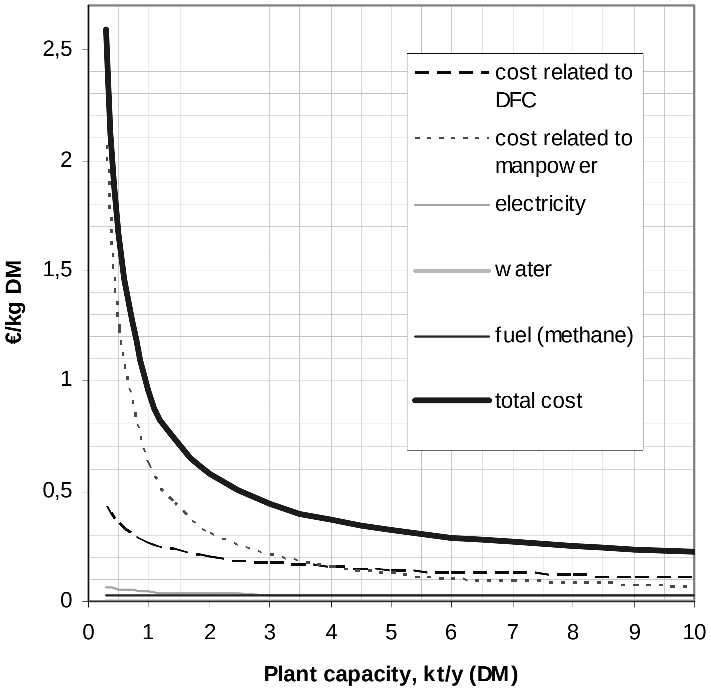


Figure 6 - Dependence of the specific biomass processing cost on the plant scale, in the case of the only steam exploded production.

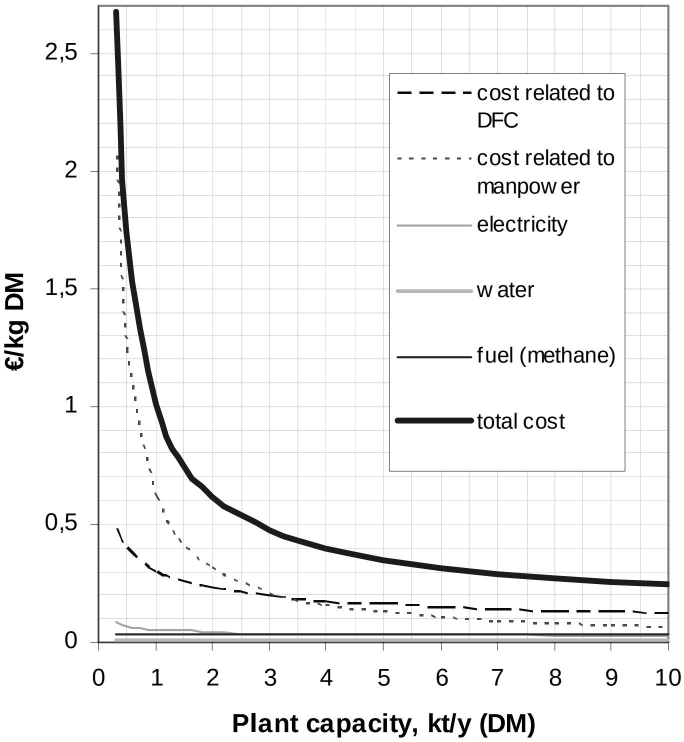


Figure 7 - Dependence of the specific biomass processing cost on the plant scale, in the case of the washed exploded production and soluble sugars recovery.

4. DISCUSSION

The mass loss after the steam explosion treatment is difficult to ascertain. Several factors as temperature, time and biomass composition, affect this datum. In the continuous steam explosion system the mass loss can be attribute to the only volatile fraction that flows out in the vapour phase out going the explosion step. Processing various lignocellulosic biomasses with the continuous steam explosion pilot plant, the mass lost was estimated by the different content of the ashes in the biomass before and after the steam treatment (it is assumed that ashes didn't subject to the thermal degradation). The overall data ranges between 2 and 8% and, considering a medium severity, the value of 5% was assumed as mass lost in the case of the Zostera and Ulva steam explosion treatment.

In the soluble material, obtained after water extraction, the sugar content was under estimated because apiose and sulfated sugars were not detected. It is assumed that the ash content is equal to salt and inorganic content. Other unknown material is a complex mix of extractives and degradation products.

The specific steam consumption ranges from 1.3 to 1.0 kg/kg$_{DM}$, these data well fit with those reported by Jollez, Chornet and Overend that studied the effect of the explosion procedures in the steam consumption and measured a specific steam consumption of 4 to 1 kg/kg$_{DM}$ operating a 4 t/h (DM) digester. In the scaling analysis it has been assumed a specific steam consumption of 1 kg/kg$_{DM}$.

The specific electricity consumption is 0.31 kWh/kg$_{DM}$ in the case I and 0.39 in the case II. 0.13 kWh/kg$_{DM}$ are imputed to the biomass feeding in the pressurized reactor. In the scaling analysis the feeder specific consumption has been kept constant, while for the other machineries it has been used an exponential factor of 0.5.

Collecting algae, the cost ranges between 0.27–0.35 €/kg$_{DM}$ (CEVA source); steam explosion process adds a cost that ranges between 0.25–1.04 €/kg$_{DM}$. Considering a selling cost from a minimum of 3 €/kg$_{DM}$ (not grounded) until a max of 20 €/kg$_{DM}$ (food quality), an income of 2–18 €/kg$_{DM}$ can be taken in account, also employing the SE process.

CONCLUSIONS

I The steam explosion treatment was optimized on Zostera to improve sugar recovery. Temperature was ranged between 160 and 180 °C, time between 60 and 300 seconds. Another variables investigated was the use of oxalic acid to precondition algae before steam explosion treatment. The load of oxalic acid was ranged between 0 and 2% respect the raw material. An experimental design of 10 runs has been elaborated and carried out. The products were characterized and data elaborated to obtain semi-empirical relationships. The mass recovery after steam treatment decreases with increasing of severity until 70% and oxalic acid did not affect it. At low temperature or time, the variation of the other two variables has not appreciable effect. The Soluble Material in aqueous phase has given indications on the crude zosterine extractability. In the explored range, the SM recovery resulted between 14 and 40% and a considerable contribute is given by oxalic acid that at high temperature has increased the SM by +8%, while at low temperature by +5%.The recoverable sugars in the aqueous extract and in the insoluble residue are resulted max at the superior extremes of the explored ranges: Temperature 180 °C, time 300 sec, oxalic acid 2%. The contribute of oxalic acid appears relevant.The high glucose content (up to 28% in the washed exploded product) make the substrate interesting to ethanol bioconversion processes.

II Zostera marina and Ulva armoricana have been analysed before and after the steam explosion (SE) treatment by means standard and on purpose developed new methods. In the Zostera, the effect of a pre-impregnation with oxalic acid has also been considered.The composition of the biomasses has been determined in terms of ash, extractives, crude zosterine and carbohydrates (rhamnose, glucose, xylose, arabinose, mannose, galactose, galacturonic acid, glucuronic acid, fucose). Elemental analysis (C, H, N, O, S) has also been carried out.The results pointed out that the yield of water soluble carbohydrates increased with the SE severity. The oxalic acid has a positive effect on the extraction yield (from 32 to 41% in the best case), so showing a synergic action with the SE treatment; both the increasing of the acidity and the metal capture (by the oxalic anion) is probably involved in this improved performance.The elemental analysis has pointed out small differences between the treated and untreated samples, with a slight increase of the ratio C/H with the SE severity. Marked differences have been found between the C content of the two raw biomasses, i.e. C_{Ulva}= 25%, $C_{Zostera}$ = 40%. Although the water soluble material (SM) increases as absolute amount with the SE severity, the highest ratio Sugars/SM in Zostera samples resulted at medium severity; probably because at high severity the extractable sugars undergo degradation reactions. The pre-impregnation with oxalic acid increases the solubility of the sugars. Glucose is the most abundant component at low severity, while galacturonic acid and xylose are the most abundant at high severity. In the Ulva, the soluble sugars yield was higher than Zostera and the non-determined material was lower. The galacturonic acid was the main carbohydrate. By analysing the water insoluble residue from Zostera, a large difference (up to 3 times) was found between the glucose determined by the standard acid hydrolysis procedure and that determined by the enzymatic tests. These results can be explained by glucose degradation in strong acid media, on the contrary, enzymatic hydrolysis is not a degradable process, but its efficiency depends on the SE severity. It can be inferred that the most suitable procedure to quantify the carbohydrates in this kind of materials is to couple a pretreatment, such as the SE, and the enzymatic digestion.

III Steam exploded Zostera is an interesting substrate for the ethanol production. The best SE conditions are resulted 180 °C, 300 s and a pre-impregnation with oxalic acid of 2% w/w. Washing with hot water, the steam exploded substrate quality increases because it enriches in cellulose percentage; moreover soluble sugars can be recovered. The cellulose of the steamed substrate can be converted into ethanol by enzymatic hydrolysis and fermentation process with overall yield up to 90%. The produced ethanol reached yield of 243g per kg of steam exploded and washed substrate and the fermentation broth is obtained at 1.5° of alcohol. Broth with a concentration of 4.7° can be obtained with overall yields of 70%, but using a high yeast dosage (12 g/l).

IV It was tested on Ulva and Zostera the effect of the steam explosion pretreatment on the yield and kinetics of biogas production. The effect of steam explosion pre-treatment is clear both in the Ulva and Zostera cases. The produced biogas was about 160 l/kg for raw Ulva, 200 l/kg for SE Ulva, 50 l/kg for raw Zostera, 120 l/kg for SE Zostera. The increasing of the biogas production can be explained with the effect of the SE that makes the fibres more accessible to the bacteria. Differently from that found in the ethanologenic processeses, the methanisation process isn't affected by inhibitors deriving from pre-treatment. The results have also pointed out differences between the two examined substrates:

SE is more effective on the zostera than on the ulva, increasing the productivity in the first of 140%, in latter of 37%;

In absolute terms, the obtainable biogas is greater in Ulva than Zostera.

A slight increase of the methane concentration in the biogas produced from the steamed substrates was also detected.

V Cost analysis was carried out on the SE process applied on the Zostera and Ulva. It is based on data collected from batch (0.5kg/cycle) and continuous plant (350 kg/h, included of the fractionation section). The treatment cost has been evaluated as a function of the plant scale. Variable and fixed cost breakdown has been detailed in the case of a 1,000 and 10,000 t/y plant.Steam explosion process costs range between 0.25 and 0.27 €/kgDM in the case of a 10,000 t/y size plant.

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