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A biorefinery concept using the green macroalgae *Chaetomorpha linum* for the coproduction of bioethanol and biogas

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ABSTRACT

An innovative integrated biorefinery approach using the green macroalgae *Chaetomorpha linum* was investigated in the present study for the co-production of bioethanol and biogas. Among three pretreatments of *C. linum* biomass, consisting of acidic, neutral and alkali ones, 3% NaOH pretreatment gave the best result in terms of thallus disintegration, biomass recovery and enzymatic digestibility as demonstrated by scanning electron microscopy and saccharification tests. The hydrolysis of *C. linum* feedstock with a crude specific enzyme preparation, locally produced from fermentation of *Aspergillus awamori*, at 45 °C, pH 5 for 30 h gave the maximum yield of fermentable sugar of 0.22 ± 0.02 g/g dry substrate. An ethanol yield of 0.41 g/g reducing sugar corresponding to about 0.093 g/g pretreated algae was obtained after alcoholic fermentation by *Saccharomyces cerevisiae*. In the integrated proposed process, mycelium issued from the fungal fermentation, liquid issued from alkali pretreatment, residual from the non-hydrolysable biomass and all effluents and co-products represent a heterogeneous substrate that feed an anaerobic digester for biogas production. GC-analysis of this later showed that the biomethane yield reached 0.26 ± 0.045 L/gVS. This study presents therefore an eco-friendly biorefining process, which efficiently coproduce bioethanol and biomethane and generate only a single waste (0.3 ± 0.01 g/g) allowing an almost complete conversion of the algal biomass.

1. Introduction

The decrease of oil resources combined to the increase of the world population and therefore the energy consumption are the main requirements for using renewable energies. Among these, biomass constitutes a renewable source of biofuel, namely bioethanol, biogas and biodiesel [1]. It represents a promising alternative for the substitution, at least in part, of fossil fuels. Indeed, the development of reliable, cost-effective and ecological processes from biomass becomes a global priority despite the two known main limits for this energetic bioconversion. In fact, the culture of lignocellulosic plants is done in detriment of cultivable land used for human consumption which is not a long term solution to the increase of population [2]. Besides, lignocellulosic biomass which is consisted of cellulose, hemicelluloses and lignin, requires mechanical, thermal and/or chemical pretreatment steps to make the cellulose accessible to enzymes during the enzymatic hydrolysis [3,4]. These pretreatment steps usually affected the cost of energetic conversion. Thus, all the research on the biological transformation of lignocellulose were interested in several issues namely finding suitable pretreatments which do not generate harmful products to the environment and fermentation inhibitors [5] and producing specific and stable enzymes with reasonable cost [6,7]. Some research were also interested in developing strains of yeasts or bacteria able to ferment simultaneously hexoses and pentoses resulting from the enzymatic saccharification as well as resistant to the various inhibitors which may be generated [8,9].

Recently, several studies are interested in finding an alternative to the use of lignocellulosic biomass. In fact, beside the use of microalgae as a source of sustainable biodiesel production [10], marine macroalgae have received considerable attention as source of third-generation biofuels [11] such as bioethanol [12–16] and biogas [17–19]. Compared to microalgae, macroalgae are multicellular plants that possess plant-like characteristics with thallus-type morphology, composed mainly of carbohydrates. They can be therefore considered as a good candidates for biofuel production like biogas, bioethanol and bio-oils [11]. Additionally, their harvesting were also easier, they represent a renewable abundant

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biomass that could be easily cultivated with low cost of collection and null environmental damage [20]. Obviously, they do not compete with land use (avoiding arable land) and water consumption, necessary for food crops [2]. Furthermore, macroalgae are characterized by a higher biomass production due to its fast growing rate in the open aquatic media [20] and does not require agricultural additives such as fertilizer and pesticides [21,22]. Moreover, they have higher photosynthetic activity than terrestrial plants [20] and they contain little or no lignin-like molecules [23].

Using macroalgae in biorefinery concepts would reduce petroleum dependence while assuring a positive environmental impact [20]. The bioethanol has been the most biofuel type produced from the macroalgae [24]. Nevertheless, the cost-effectiveness of biorefinery concept which is based on the production of bioethanol is debatable for the low cellulose content (15–25%) and the seasonal and environmental variation of macroalgae which influences its biochemical composition including the content of cellulose principal source of fermentable sugars [25].

Thus, the objective of our study is to develop a novel integrated biorefinery concept based on the co-production of both bioethanol and biogas from the green macroalgae *Chaetomorpha linum* with one coproduct. *C. linum* is very abundant in the coasts of Tunisia but it is not very valued. The feasibility of different stages of the process such as pretreatment of macroalgae, alcohol fermentation and anaerobic digestion was demonstrated. In this work environmental friendly cell-wall degrading enzymes, locally produced, were used for the saccharification of *C. linum*.

2. Materials and methods

2.1. Biological materials

The green macroalgae *C. linum* was collected in September 2013 from the shores of Tunis lagoon (GPS: 36.813095, 10.192673, salinity: 33.8 psu) suffering from eutrophication problem. A bioremediation of this ecosystem could be attempted using these stranded algae as feedstock of a biorefinery process. Samples were washed, dried, finely ground and stored until they were used.

Aspergillus awamori (NBRC 4033, Osaka, Japan) was maintained at 4 °C in potato dextrose agar plates. The spores were collected in 4 mL of sterile water containing 0.1% Tween 80 and transferred into a 250 mL Erlenmeyer flask containing 75 mL of medium.

Baker's yeast (*Saccharomyces cerevisiae*) was purchased from the local market (la Patissiere Company). The strain stored at −20 °C in 25% glycerol was firstly purified by subculture on YPG (yeast peptone glucose) agar medium. One purified colony was transferred in YPG broth to start liquid precultures for 12 h with 180 rpm agitation on rotary shaker. Microscopic observations were performed to ensure the presence of *S. cerevisiae* and its purity. The culture was used at 10% v/v as inoculum for alcohol fermentation.

2.2. Cell wall degrading enzymes production

Cell wall degrading enzymes production was carried out in triplicate with a batch fermentation of *A. awamori* (NBRC 4033) in mineral medium according to Mandel and Weber [26]. Mandels' salts solution (0.3 g L^{-1} urea, 1.4 g L^{-1} $(NH_4)_2SO_4$, 2.0 g L^{-1} KH_2PO_4, 0.3 g L^{-1} $CaCl_2$, 0.3 g L^{-1} $MgSO_4$, 0.25 g L^{-1} yeast extract, 0.75 g L^{-1} peptone, 5 mg L^{-1} $FeSO_47H_2O$, 20 mg L^{-1} $CoCl_2$, 1.6 mg L^{-1} $MnSO_4$ and 1.4 mg L^{-1} $ZnSO_4$) supplemented with 0.5% (w/v) ground *C. linum* as carbon source was used. The pH of the medium was adjusted to 5 with 50 mM sodium acetate buffer. The flasks were autoclaved for 30 min at 120 °C and then inoculated with the *A. awamori* preculture. After 8 days incubation at 40 °C the cocktail of enzymes was obtained by simple filtration on gauze filter followed by ultrafiltration on 10 kD membrane cut-off.

2.3. Pretreatment conditions

C. linum were pretreated by thermohydrolysis carried out for 20 min at 120 °C in an autoclave (1.5 bars) without catalyst for neutral pretreatment and in the presence of 3% NaOH and 0.6% H_2SO_4 for alkali and acid pretreatment respectively.

Pretreated biomass was recovered by centrifugation (solid residue) and used at 4% (w/v) for the enzymatic saccharification after pH adjustment with sodium acetate buffer 100 mM. The supernatant corresponding to the liquid issued from pretreatment was also recovered and served to feed the anaerobic digester for the production of biogas.

For the determination of the residual mass, pretreated and filtered samples were dried in an oven at 104 °C overnight and then weighed by precision balance. Experiments were done in triplicate.

2.4. Biomass saccharification and alcohol fermentation

Enzymatic hydrolysis was carried out with 10% of alkali pretreated *C. linum* at pH 5. The saccharification reactions were conducted in triplicate in a laboratory incubator at 150 rpm and 45 °C for 30 h.

Ethanol production was studied in triplicates using the broth of enzymatic saccharification of *C. linum*. The saccharification reaction products were concentrated by evaporation at 60 °C for 12 h to reduce the water content and make the reducing sugars content of about 40 g/L. The saccharification broth was sterilized by filtration (0.2 μm filter membrane) and added to Yeast extract Peptone medium (YP) (10× concentrate) with the proportion (9/1). The prepared medium was inoculated with 10% v/v of fresh culture of *S. cerevisiae* and incubated at 28 ± 2 °C on an orbital shaker with a shaking speed of 150 rpm.

After 48 h of fermentation, the ethanol concentration was assayed by HPLC. The ethanol is recovered at high purity by conventional distillation at 60 °C. Vinasse (residue after fermentation / distillation) was recovered to feed the stage of anaerobic digestion.

2.5. Anaerobic digestion

The anaerobic digestion was achieved in duplicate in a batch stirred anaerobic reactor with a working volume of 0.5 L. The reactor was operated under mesophilic conditions (38 ± 1 °C) during 30 days. Over the anaerobic digestion heterogeneous substrate corresponding to all liquid, solid and gaseous effluent produced in the different stages of the manufacturing process of bioethanol was recovered. Inoculation with active methanogenic bacteria with an inoculum ratio of 50%/50% (v/v) for the digestion of *C. linum* was achieved. Initial anaerobic sludge used as inoculum was collected from an active digester located in a municipal wastewater treatment plant of Chotrana (Tunisia). The biogas produced was collected daily in plastic bags at room temperature. The total volume was later determined with wet gas-meter (Ritter, Germany). The methane content in the biogas was measured using a FID–PID Unichrom gas chromatograph.

2.6. Biochemical characterization of the cellulase crude extract

The optimal pH of the endoglucanase (CMCase) was determined using the following buffers at 100 mM: Glycine–HCl (for pH from 2.2 to 3), sodium acetate (for pH from 4.0 to 6.0), potassium phosphate (for pH from 6.0 to 8.0) and glycine–NaOH (for pH from 8.0 to 10.0). For temperature stability, the enzyme was pre-incubated in the standard buffer (sodium acetate pH 5) at 40, 50, 60 and 70 °C for 30 min and the activity was thereafter assayed by DNS method under the standard conditions.

2.7. Enzyme assays

Filter paper assay was used to estimate total cellulase activity in the crude extract according to Ghose [27] and expressed as filter paper units (FPU).

Carboxymethyl cellulase (CMCase), β-glucosidase, xylanase, β-xylosidase and α-L-arabinofuranosidase activities were assayed using respectively as substrates 1% (w/v) carboxymethyl cellulose, 0.5 mM p-nitrophenyl β-D-glucoside (pNPGlc), 1% (w/v) xylan, 0.5 mM p-nitrophenyl β-D-xyloside and 1 mM p-nitrophenyl α-L-arabinofuranoside at pH 5 and 45 °C [28]. One unit (U) of each enzyme activity is defined as the amount of enzyme which produces 1 μmole product in the reaction mixture per min under the assay conditions used.

Proteolytic activity (protease) was assayed as described by Abidi et al. [29] using 5% azocasein as substrate .The reaction was carried out at 50 °C and pH 7.

The dosage of the total protein content was carried out according to the method of Bradford [30]. All tested activities were performed in triplicate and expressed as specific activities (U/mg of proteins) and the results were presented as mean ± SD (standard deviation).

2.8. Analytical methods

Estimation of total reducing sugar in the enzymatic hydrolysate of biomass was done by DNS method [31]. Dry matter and ash content were determined according to standard APHA methods [32] by drying the macroalgae at 105 °C (24 h) followed by incineration at 550 °C (2 h). Total fiber contents were determinate according to the AOAC enzymatic–gravimetric method of Prosky et al. [33]. Cellulose was extracted using the method described by Jmel et al. [34], dried at 105 °C and weighed for the determination of the cellulose content. The content of lipid was determinate using the protocol described in the standard NF V 03-713 [35]. Measurement of total Kjeldahl nitrogen (TKN) was carried out using the normalized APHA method [32]. The conversion factor 4.92 was used to estimate the protein content ($N \times 4.92$).The results of different component of *C. linum* were expressed in percent of dry weight and were presented as mean ± SD (standard deviation of triplicates).

The carbohydrate content (glucose, xylose, arabinose and rhamnose) of *C. linum* was determinate in duplicate using the strong acid hydrolysis protocol adapted from Effland [36]. Dried and milled macroalgae (100 mg) were hydrolyzed with 12 M H_2SO_4 for 2 h at room temperature, then diluted to reach a final acid concentration of 1.5 M and kept at 100 °C for 3 h. The mixture was filtered through paper fiberglass (GF/F, WHATMAN). Analysis of glucose, xylose, arabinose and rhamnose were done by using high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) on a Dionex system (Dionex, CA, USA) equipped with a CarboPac PA-1 column (Dionex, 250 × 4.5 mm) and a suitable guard column. Flow rate was 1 mL/min, and the applied gradient was 1 M sodium acetate in 0.1 M NaOH. Commercially available D-glucose, D-xylose, L-arabinose and L-rhamnose were used as standards, and fucose was the internal quantitative standard.

The zymogram analysis of extracellular cellulases was performed by 10% (w/v) polyacrylamide gel according to Laemmli [37] with some modifications. The crude enzyme samples mixed with the same volume of loading buffer were boiled at 100 °C for 4 min and subjected to SDS–PAGE stained with Coomassie Brilliant Blue R-250. Zymogram with CMC was achieved as described by Sun et al. [38] with some modifications, and enzyme samples were denatured with 3% SDS in Tris–HCl buffer (0.03 M, pH 6.8). Samples were treated at 80 °C for 10 min to avoid the possibility of incomplete denaturation. After separation of the enzyme samples by SDS–PAGE (0.15% CMC was added into the gel), the gel was washed twice at room temperature with the wash solution [50 mM acetate buffer and 25% isopropanol (pH 5)] to remove the sodium dodecyl sulfate. The enzyme components were renatured in a 50 mM acetate buffer (pH 5) containing 5 mM β-mercaptoethanol by stirring the gel overnight at 4 °C. Then the gel was washed by 50 mM acetate buffer (pH 5) for 1 h and incubated at 50 °C for another 30 min. The gel was stained in a 0.1% (w/v) congo red solution for another 30 min before destaining with l M NaCl, then pale red hydrolysis zones appeared against a red background.

Thin layer chromatography (TLC) analysis of algal hydrolysate obtained following the enzymatic saccharification was conducted on silica plates (K60 Merck) using a mobile phase composed of n-butanol/acetic acid/H_2O (1.5/1.5/1 v/v/v). After migration the plates were dried and pulverized with a solution of sulfuric acid 20% v/v. Spots were revealed by incubating the plates at 105 °C for 10 min.

The HPLC analysis of either saccharification products or bioethanol formation, were done using Prontosyl C18 column (250 × 4 mm, 5 μm, ICS France) at 70 °C with refractive detector. The mobile phase was H_2SO_4/water at 0.1% (v/v). The flow rate was 0.5 mL/min and the injection volume was 20 μL. Commercially available D-glucose, D-xylose, L-arabinose and L-rhamnose were used as standard sugars.

3. Results and discussion

3.1. A. awamori culture and specific enzyme production

Filamentous fungi are well known microorganism of decomposition of organic matter in general and of cellulosic substrates in particular as reported in many studies [39]. *A. awamori* was used in this study as a source of hydrolytic enzymes capable of hydrolyzing the green macroalgae *C. linum* and liberating fermentable sugars. Our strategy consisted of culturing *A. awamori* in submerged minimum medium supplemented by the ground *C. linum* algae as the sole carbon source. Table 1 showed that cellulolytic (endoglucanase and β-glucosidase), hemicellulolytic (xylanase, β-xylosidase and α-L-arabinofuranosidase) and proteolytic activities were induced and found in the fermentation broth. This demonstrated that *A. awamori* was able to hydrolyze the complex structure of the *C. linum* macroalgae and therefore metabolizing the organic nutrients to ensure a correct growth. This strategy of induction permitted the obtaining of specific enzymes to the used-carbon source, ensuring subsequently the success of saccharification experiments. The main specific activities of cell wall degrading enzymes after six days of culture are summarized in Table 1.

The time courses of Endo-α-(1-4)-glucanase (CMCase) and FPase (filter paper degradation: Total cellulase activity) activities

Table 1
Measurement of specific activities of cell wall degrading enzymes before ultrafiltration.

Enzyme class	Enzyme activity	Specific activity (U/mg)[a]
EC 3.2.1.4	Endo-β-(1-4)-glucanase (CMCase)	8.01 ± 0.27
EC 3.2.1.21	β-glucosidase	9.93 ± 0.31
EC 3.2.1.8	Endo-β-(1-4)-xylanase	2.26 ± 0.13
EC 3.2.1.37	β-xylosidase	1.1 ± 0.04
EC 3.2.1.55	α-L-arabinofuranosidase	0.83 ± 0.06
EC 3.4	Protease[b]	0.9 ± 0.04

[a] U/mg is the number of μmol produced (glucose equivalent in the case of CMCase activity, equivalent xylose in the case of xylanase activity, and para-nitrophenol in the case of α-glucosidase activities, α-xylosidase and α-L-arabinofuranosidase) released per minute per 1 mg of total protein.

[b] 1 U protease corresponds to 0.1 OD at 440 nm using azo-casein as a substrate.

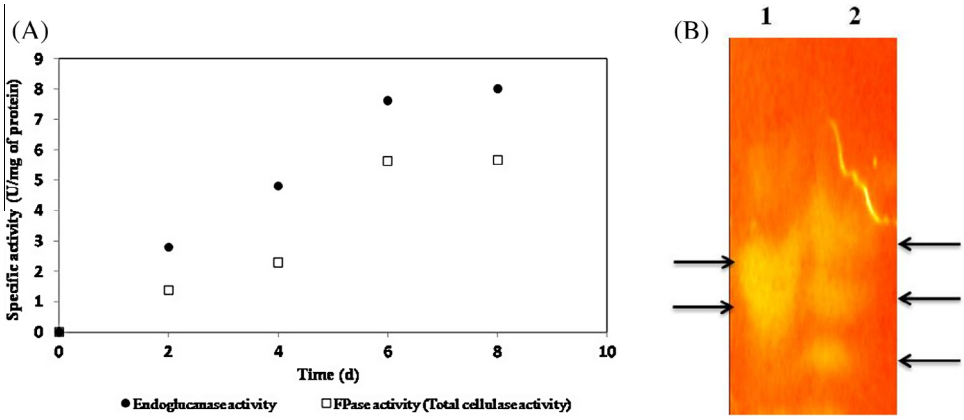

Fig. 1. (A) Time course of enzyme production by *Aspergillus awamori* using *C. linum* as an induction source (●) Endoglucanase activity (□) FPase activity (Total cellulase activity). Assays were done in triplicate. Measurement standard error did not exceed 5%. (B) Zymogram Analysis of crude enzymatic extracts obtained respectively from cultures on (1) filter paper as conventional inducer and (2) *C. linum* as a specific inducer showing differential induction and a specific electrophoretic cellulase profile for *C. linum*. Arrows indicate positions of the different isoforms.

were shown in Fig. 1A. Both enzymes were extensively secreted the first six days, where maximal activities of 8.01 and 5.63 U/mg were respectively reached. The secretion of these activities remained constant thereafter until depletion of the medium. To demonstrate the specificity of the cellulases produced, *A. awamori* were also cultivated under the same conditions with ground filter paper used as a sole carbon source and conventional inducer of cellulases. Zymograme analysis of the crude extracts (Fig. 1B), issued from *C. linum* and filter paper as inducers showed two different electrophoretic profiles. At least three isoforms can be observed with *C. linum* as carbon source. The multiplicity of isoforms and the specificity to the carbon source is a known phenomenon that allows to filamentous fungi to acclimate on the media composition by secreting other enzymes, and/or modifying them at transcriptional or translational levels [40–42]. It is also important to note that this faculty of *A. awamori* to produce specific enzymes could be improved by Solid State Fermentation (SSF), which is known to can increase the cellulase activities [43].

The obtained cellulase crude extract, issued from the culture with *C. linum* as inducer, was biochemically characterized in terms of temperature and pH. The optimal temperature and the optimal pH of the endoglucanase (CMCase) were 45 °C and 5 respectively. CMCase activity was very stable since its half-life time ($T_{1/2}$) derived from plots of residual activity $Log_e(A/A_0)$ vs time was found 11 h at 45 °C. While these results are similar to known commercial cellulases [44], it's important to denote the specificity of this preparation to green algae composition and its low-cost of obtaining. These two criteria represent a success keys to perform a down-stream enzymatic-based process.

3.2. Global composition of C. linum

The global chemical composition of *C. linum* was summarized in Table 2. It is important to report that the fiber content was found at 27 ± 1.8% containing 21 ± 1.2% cellulose based on dry weight. *C. linum* had an important content of fibers (cellulose, hemicelluloses...) compared to other macroalgae, which made it a good source for bioenergy production [14]. In fact, as shown by fine chemical composition of the carbohydrate content (Table 3), *C. linum* contained glucose, xylose/arabinose and rhamnose resulting from the degradation of cellulose, hemicelluloses and ulvan respectively. These results are in agreement with several studies which demonstrated that the macroalgae are potentially good sources of polysaccharides, proteins and fibers [45–47]. The ash content of 30.5 ± 4.2% (Table 2) seems to be high, but it was in accordance with the usual values known for green algae ranging from 11% to 34% on dry weight basis [48].

Table 2
Biochemical composition of *Chaetomorpha linum*.

Proximate composition	Relative % on dry weight basis
Dry matter	83 ± 1.3
Water	17 ± 0.5
Fibers	27 ± 1.8
Protein	8.6 ± 0.47
Lipid	2.6 ± 0.12
Ash	30.5 ± 4.2

Table 3
Carbohydrate chemical composition of *Chaetomorpha linum*.

Carbohydrate content	Glucose	Xylose	Arabinose	Rhamnose
Relative % on dry weight basis	26.2 ± 2.1	2.29 ± 0.25	0.56 ± 0.03	0.71 ± 0.06

Previous investigation of the detailed chemical composition of *C. linum* also showed high content in polysaccharides. It was consisted of 34–38 g glucan, 6 g xylan, 9–10 g arabinan, 7 g non hydrolysable organic components, 21–23 g ash, 14 g pectin and 6 g wax per 100 g dry matter [14]. However, it's important to note that the chemical composition of macroalgae presents a great variability, which is related to several abiotic factors, mainly environmental ones such as salinity, water, temperature, light, and seasonal variation of nutrients [49,50].

3.3. Pretreatment and saccharification

Three types of pretreatment have been tried, neutral (with H_2O), alkali (with 3% NaOH as catalyst) and dilute acid (0.6% H_2SO_4 as catalyst). The determination of the residual mass content in the pretreated algae (Table 4) showed that the acid pretreatment is accompanied by a significant loss of solid matter while the hydrothermal one was the best on pretreated biomass recovery. The NaOH pretreatment assayed on *C. linum* at low concentration gave interesting results. It permitted to recover a significant residual pretreated biomass (Table 4) compared to acid pretreatment, while increasing the enzyme accessibility by significant destructuration of the cell wall (Fig. S1). Indeed, it's known that the acid pretreatment results in a nearly complete solubilization of hemicelluloses which explain the low residual mass generally achieved [3]. This pretreatment, widely employed for

Table 4
Effect of different pretreatments on the residual content of *Chaetomorphae linum* and saccharification yields.

Algae	Pretreatment	Residual dry mass (%)	Saccharification yield (g/L)[a]
Chaetomorphae linum	Neutral (H_2O)	92 ± 2.88	8.6 ± 0.14
	Acid (H_2SO_4 at 0.6%)	56 ± 4.01	5.8 ± 0.17
	Alkali (NaOH at 3%)	75 ± 3.54	11.2 ± 0.32

[a] The saccharification yields were expressed by g of reducing sugar per liter produced after 12 h of reaction.

lignocellulosic biomasses, is used for efficiently removing hemicelluloses by breaking ether bonds in lignin/phenolics–carbohydrates complexes without dissolving lignin [51]. Its use with macroalgae thallus is less common regarding both the atypical structure and the composition of this kind of biomass. In fact, as shown by Trivedi et al. [15] this type of pretreatment leads to low saccharification yields of green macroalgae *Ulva fasciata* [15]. However, alkali pretreatment leads to the saphonification of esters of the uronic bonds between hemicelluloses and lignin, swells the fibers and increases the pore size, facilitating the diffusion of the hydrolytic enzymes. In the case of macroalgae it also permit to remove ulvan [52]. On the other side, tests of saccharification performed on the pretreated biomasses for 12 h showed that the NaOH pretreatment gave the highest value of reducing sugars (Table 4), suggesting that it could be considered as the suitable choice for *C. linum* biomass. This later was hydrolyzed in a larger volume with a specific crude enzyme preparation as described above. A time course of this saccharification reaction was assessed as shown by Fig. 2A. The content of reducing sugars increased quickly at the beginning stage (5–10 h), and the highest concentration (12 g L^{-1}) was obtained after 30 h reaction. This reducing sugar production was accompanied by radical change of the reaction appearance (Fig. 2C). Indeed, the insoluble filamentous thallus of *C. linum* was completely dissolved by the enzymes to give homogenous solution. TLC analysis of the final reaction products showed that glucose corresponded to the major product (Fig. 2B). This finding was also confirmed by HPLC analysis (Fig. 3). Compared to retention times of standard monosaccharides (Fig. S2),

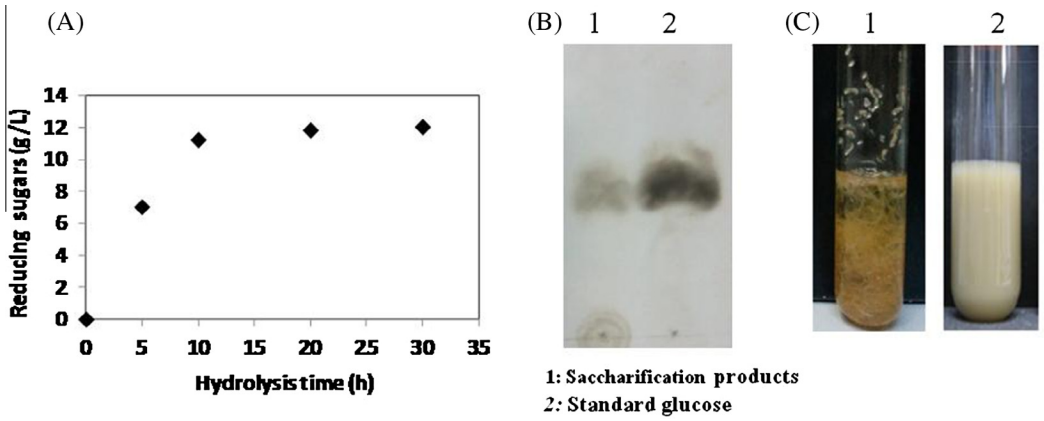

Fig. 2. (A) Time course of enzymatic saccharification of alkali-pretreated *C. linum* biomass using *Aspergillus awamori* crude enzymatic extract obtained with *C. linum* as inducer. Experiments were done in triplicate. Measurement standard error did not exceed 10%. (B) TLC analysis of the saccharification products (1) using glucose as standard (2). (C) Appearance of the alkali-pretreated algae before (1) and after (2) enzymatic saccharification.

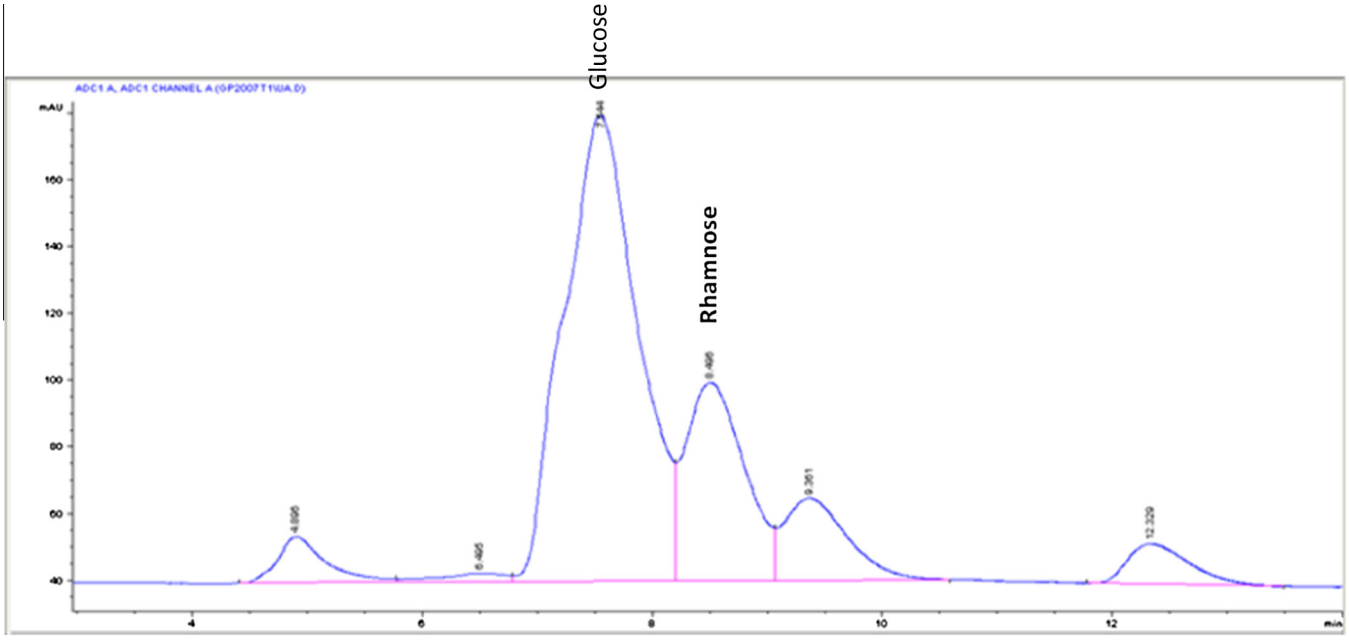

Fig. 3. High performance liquid chromatography analysis (HPLC) of the enzymatic saccharification products.

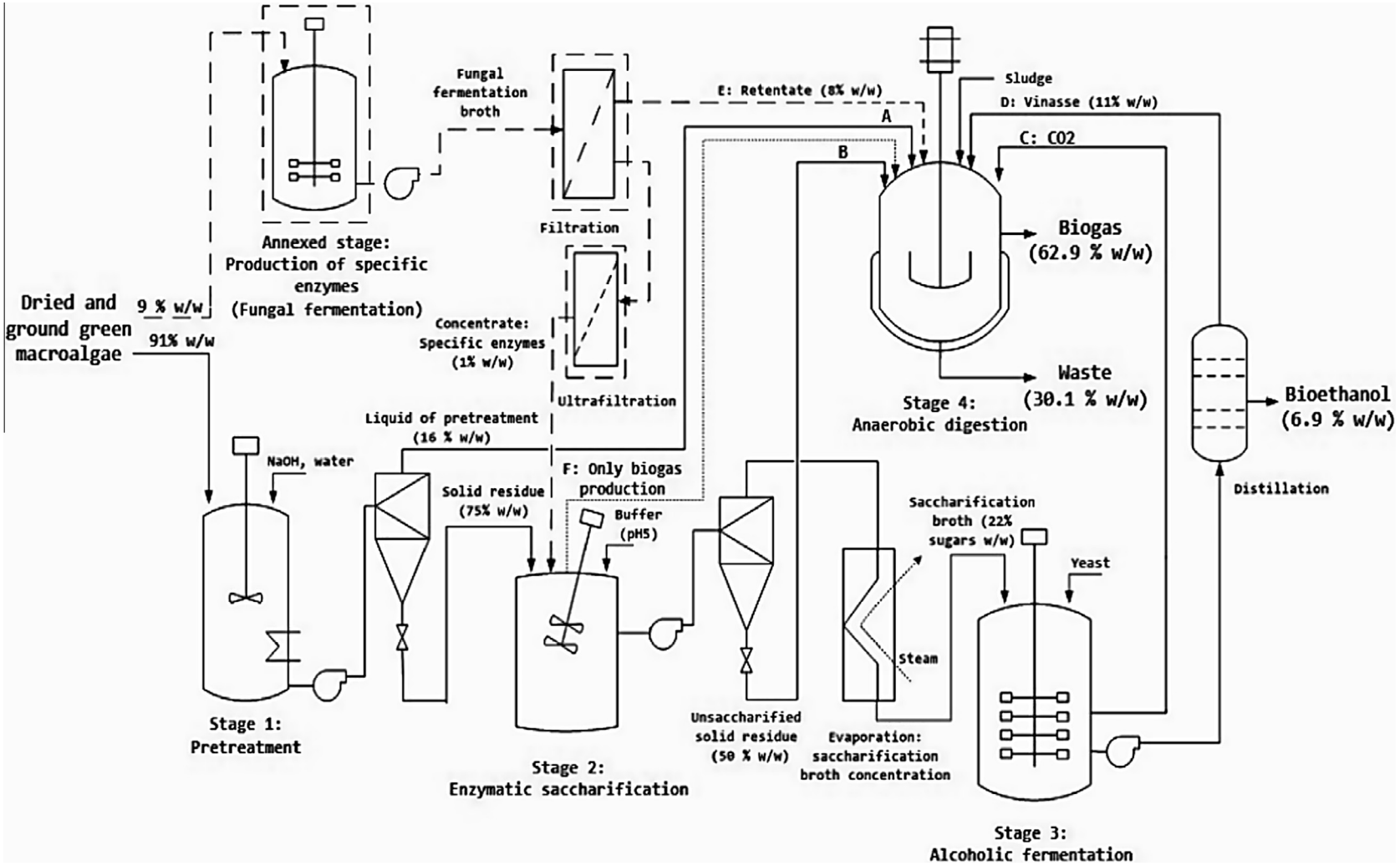

Fig. 4. Flow diagram and main inputs/outputs of the units in the proposed biorefinery concept aimed at the coproduction of bioethanol and biogas from green macroalgae *C. linum* biomass. The inputs/outputs were expressed by relative percentage on dry weight of initial algae basis (w/w dry matter of initial algae). Continuous and discontinuous lines were used for main stages and annexed stages respectively. Inputs for anaerobic digestion: (A) Liquid of pretreatment, (B) unsaccharified solid residue, (C) CO_2, (D) vinasse and (E) retentate (mycelium issued from fungal fermentation). (F) Alternative path for only biogas production.

saccharification products consisted mainly of glucose as major product and rhamnose.

The total amount of reducing sugars released from 1 g of algae feedstock catalyzed by the crude enzyme was 0.22 ± 0.02 g/g^1 dry substrate. Similarly, in a previous study the saccharification of the green macroalgae *U. fasciata* with a commercial enzyme Cellulase (fluka) for 36 h at 45 °C gave maximum yield of sugar 0.20 ± 0.014 g/g dry weight [15].

3.4. Development of an integrated process for C. linum conversion to bioethanol and biogaz

After studying the feasibility of different steps including specific enzymes production, pretreatment and saccharification of *C. linum*, we have developed a process that integrates all the stages of a biorefinery concept to co-produce both bioethanol and biogas from this macroalgae with minimum waste. This diagram consisted of four main stages namely pretreatment, enzymatic saccharification, alcoholic fermentation and anaerobic digestion with annexed stage of local enzymes production/preparation. The detailed flow diagram of this process is illustrated in Fig. 4. Intermediate units of filtration, ultrafiltration, centrifugation, evaporation and distillation were also presented in this diagram.

The first stage concerned the pretreatment of dried macroalgae (stage 1, Fig. 4) at 120 °C and 1.5 bars, in the presence of alkaline catalyst at low concentration (3% NaOH). This pretreatment allows the disintegration of the walls of algal thallus in order to increase the accessibility of polysaccharides and decrease the intracellular concentration of ulvan and minerals that move in the pretreatment liquid causing less inhibition for the subsequent saccharification and fermentation. Conditions of temperature and pressure applied in this case remain mild conditions that allow saving energy and chemical reagents.

A step attached to the process allows the production of specific hydrolytic enzymes (annexed stage, Fig. 4). This production is made by fermentation of selected filamentous fungi "*A. awamori*" having the ability to grow specifically on *C. linum*, used as carbon source. This fungus is used to produce a cocktail of cellulases, hemicellulases, proteases, etc. Indeed, as mentioned above, specific extracellular hydrolases were efficiently produced (Table 1) during this fermentation. These enzymes are recovered by simple filtration (to remove the mycelium) followed by an ultrafiltration step on 10 KDa cutoff membrane to concentrate the enzyme preparation. Enzymes are thereafter introduced directly into the stage 2 of saccharification (Fig. 4). This fungal fermentation allowed not only the production of inexpensive specific enzymes but also enriched the anaerobic digestion by 8% (w/w) of biomass which enhanced the biogas production and increased the efficiency of the process.

The second stage is the enzymatic saccharification of alkali pretreated biomass, at pH 5 (stage 2, Fig. 4), considered as a relative optimum pH of hydrolases of the *Aspergillus* genus [53].

A fermentation step (stage 3, Fig. 4) uses the saccharification broth obtained in stage 2 and concentrated by evaporation. After fermentation the ethanol is recovered by simple distillation at 60 °C by a conventional system. In this step CO_2 (gas discharge) from the fermentation is recovered by a system of pipes connected to a sealed bag with independent input/output used to power the digester (input C, stage 4) in a continuous way. The residue after distillation (vinasse) also serves to feed the anaerobic digester (input D, stage 4).

Finally the anaerobic digestion step (stage 4, Fig. 4) aims to produce biogas biomethane basically. This step is carried out with heterogeneous substrate made of all solid effluents, liquid and gaseous phases of the preceding and appendices (inputs A, B, C, D and E, Fig. 4). This substrate consists of the liquid of pretreatment containing matrix sugars of seaweed, hemicelluloses, minerals and all that hot water soluble, the solid residue of un-saccharified pretreated biomass, CO_2, vinasse (yeast biomass after alcoholic fermentation and distillation), and mycelium from annexed step of fungal fermentation. This later heterogeneous substrate is mixed with a suitable inoculum of methanogenic bacteria to obtain the maximum biogas and the maximum degradation. Unconverted substrate is the only co-product of the entire process. In this step, inhibition that could be exerted by minerals and sulfated sugars is not manifest since they are diluted in the total effluent. In the other side, the solid waste including the mycelium and vinasse contribute to the stimulation of anaerobic digestion.

The yields of bioethanol and biomethane produced from the energetic bioconversion of *C. linum* using this process is presented in Table 5. The conversion of *C. linum* provided 0.093 g/g pretreated

Table 5
The balance sheet of the *Chaetomorphae linum* energetic bioconversion.

Macroalgae	Bioethanol yield		Biomethane yield		Waste	
	g/g pretreated algae	% g algae	L/gVS	% g algae	g/g algae	% g algae
Chaetomorphae linum	0.093 ± 0.003	6.9	0.26 ± 0.045	62.92	0.301 ± 0.01	30.18

Table 6
Comparison of ethanol and methane yields reported for different macroalgal feedstocks with present study.

Macroalgae	Part used for bioconversion	Pretreatment conditions	Sugar released (g/g)	Ethanol yield (g/g sugar)	Methane yield (L/gVS)	References
Chaetomorphae linum	Whole biomass	Alkali + enzyme	0.22	0.41	0.260	Present study
Laminaria digitata	Whole biomass	Oven-drying	NA	12.3 μL/gDS	_	[60]
		Freeze-drying	NA	_	0.257	
Ulva rigida	Whole biomass	Enzymatic	0.384	_	0.626 L/g CODint	[61]
Fucus vesiculosus	Whole biomass	Thermo-acidic	NA	_	0.116	[62]
Ulva lactuca	Whole biomass	_	NA	_	0.241	[18]
Palmaria palmata	Whole biomass	_	NA	_	0.279	[18]
Saccharina latissima	Whole biomass	_	NA	_	0.209	[18]
Chaetomorpha linum	Whole biomass	Wet oxidation (200 °C) + SSF	0.74	0.44	_	[14]
		Ball milling (18 h) + SSF	0.36	0.44	_	
Ulva fasciata	Whole biomass	Hot buffer + enzyme	0.205	0.45	_	[15]
Kappaphycus alverzii	Whole thallus	Acidic	NA	0.369	_	[63]
Gelidium amansii	Whole biomass	Dilute acid hydrolysis	0.422	0.38	_	[22]
Kappaphycus alverzii	Granule	Acidic	0.306	0.4	_	[13]
Saccharina japonica	Whole thallus	Thermal acid hydrolysis	0.456	0.169	_	[64]
Laminaria japonica	Whole biomass	Acid + Enzyme	0.376	0.41	_	[21]

algae and 0.26 L/gVS for bioethanol and biomethane respectively with 0.3 g/g of waste (Table 5). It's important to note that seasonal variability and environmental constraints could affect the chemical composition of *C. linum* and therefore these obtained values. The proposed process presents the advantage to be flexible depending to the algal biomass composition. As shown in Fig. 4, if the cellulose content or cristallinity affects the yield of saccharification, the bioethanol production could be switched off (path F, Fig. 4) to only produce biogas.

To better evaluate and discuss the bioethanol and biogaz production from our integrated process, a comparison study was carried out with different macroalgal feedstock (Table 6). Although, it was a coproduction from the same substrat, this process provided comparative yield of bioethanol (0.41 g/g sugar) and a maximum yield of biomethane (0.26 L/gVS) compared to values reported earlier, especially from a similar green macroalgae *U. fasciata* [15]. All previous studies were interested to produce either bioethanol or biomethane. However, this work presents innovative eco-friendly process which incorporates a set of technologies related to the processing of green macroalgae *C. linum* in a way to generate only a single waste allowing an almost complete conversion of the algal biomass. This study opens also possibility to conceive different biorefinery scenarios depending on the composition of algae for the efficient use of this biomass.

A basic economic analysis is useful to give more evaluation of the proposed process. Typically, an economic approach of a biorefinery concept can be focused on three key cost contributors namely biomass feedstock (including its transportation), hydrolysis enzymes and other operating costs [54].

For the cost of the biomass, considered as the highest one in algal biorefineries [55], we have in our case no culture constraints, since the algae used corresponded to eutrophication products stranded on shores. Furthermore, Konda et al. [54] showed that the overall cost of algal biomass and its transport was estimated at 24% (around $100/MT) of the biorefinery total cost. Otherwise, knowing that the transportation cost could be estimated at 10–15% of the cost of biomass feedstock [56,57], we can deduce that the cost of biomass in an algal biorefinery could be estimated at 14% of the biorefinery total cost. So, the proposed process will save about 24% of the biorefinery total cost if this later will be installed locally to meet the local fuel consumption of rural population [58]. This saving can be estimated at 14% of the total cost in the case of a distant biorefinery with transport.

Regarding the cost of enzymes, which was estimated at about $10.14/kg of protein corresponding to 22% of the biorefinery total cost [54]. Our process has the advantage of using locally produced enzymes that can save this cost. The other operating costs including ones of operation units could be comparable to those of a conventional lignocellulosic biorefinery [56].

From the energetic point of view, it was previously mentioned that the cost of energy production from macroalgae is typically belonging to the interval 5–60 $/GJ on the basis of macroalgal biofuels life cycle assessment [59]. Concerning the proposed process in this work, we estimate that its energetic cost could be situated at the lower range of the mentioned interval given two main reasons: firstly because the energetic efficiency is higher related to the production of two kind of biofuel (bioethanol and biogas) with minimum waste, and secondly given the energetic saving related to the absence of macroalgae cultivation and transportation. Finally, regarding the fact of the novelty of this process and the lack of detailed economic and energetic data dealing with integrated process from macroalgae [55], a complete techno-economic study with detailed modeling and simulation seems to be necessary for a precise determination of both total efficiency and basic economics of the proposed process.

4. Conclusion

This study presents a proof of concept demonstrating the feasibility of the co-production of both biogas and bioethanol from the entire green macroalgae *C. linum* using an eco-friendly process with minimum waste. The proposed process was composed of three main stages of pretreatment, saccharification and fermentation aimed at the bioethanol production, one annexed stage for locally cell-wall degrading enzymes production, and one main stage of anaerobic digestion for biogas production including all effluents and co-products issued from these latest stages and intermediate operations. In our investigation locally produced enzymes were used for the biomass saccharification, which reduce the cost of the bioconversion and increase the efficiency of the proposed process. The yields obtained are 0.093 ± 0.003 g/g pretreated algae and 0.26 ± 0.045 L/gVS for bioethanol and biomethane respectively with only 0.3 ± 0.01 g/g of waste. Optimization of some steps of this integrated process and a complete techno-economic study are therefore necessary to envisage the scale-up of this proposed concept.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enconman.2016.04.046.

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