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Production of Free Sugars by Enzymatic Hydrolysis of *Dictyota dichotoma*

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It is well-known that brown macroalgae can be used as a source of valuable sugars with different biological activities, but its polymeric macrostructures should be hydrolyzed for obtaining an acceptable yield. This study deals with the production of free sugars by the enzymatic hydrolysis of optimized biologically pretreated brown seaweed. The biological pretreatment was carried out using solid-state fermentation (SSF) with a white root fungus species (*Aspergillus awamori*). For this purpose, different temperature growth conditions (30ºC and 50ºC) and inoculum concentrations (10^6; 10^7 and 10^8 spores/g of dried solid) were tested for 20 days of the SSF process. In addition, different dosages of commercial hydrolytic enzyme cocktail Cellic® Ctec2 were tested for enzymatic hydrolysis (EH) of *D. dichotoma* at a solid/liquid ratio of 2.2% w/v. Finally, biologically pretreated samples at optimum conditions (30ºC and inoculum size of 10^8 cel/mL) were hydrolyzed with commercial Cellic® Ctec2 at the optimum dosage (112FPU/g). The maximum reducing sugars concentration measured in the hydrolysates was 80 g/kg biomass. Further investigations increasing the solid-liquid ratio and reducing the enzyme dosage are necessary for the complete optimization of sugar production from *D. dichotoma*.

1. Introduction

Brown seaweeds (Phaeophyta) have been tested in Science to produce different polysaccharides such as alginates, laminarin or fucoidan with different functions (Rabanal et al., 2014). *Dictota dichotoma,* also named as *Dictyota apiculata*, *Dictyota vivesii, Ulva dichotoma* and *Zonaria dichotoma,* is an autochthonous alga from Spain which has been registered in provinces such as Asturias, A coruña, Pontevedra, Alicante, Murcia or Cádiz but also in other parts of Europe, South Africa and Bermuda. This specie has been previously used for extracting interesting compounds such as fucoidan, laminarin and its sulfated derivative, which show different biological activities like anticoagulant, antitumoral, antiviral, etc. (Usoltseva et al., 2018). However, in the last ten years, different species of macroalgae have been used as substrate for enzymatic hydrolysis (EH) to produce monosaccharides which can be fermented to other high value-added products. In the case of brown macroalgae, the most used species are *Sargassum sp* (Azizi et al., 2017)*, Laminaria japonica* (Hong et al., 2014) and *Saccharina latissima* (Sharma and Horn, 2016). In this sense, different enzymatic cocktails have been tested (Hong et al., 2014 and Jena et al., 2022) where Cellic® Ctec2 has been extensively used for EH of brown seaweed. As an example, Jena et al., (2022) applied Cellic® Ctec2 at 50ºC at 3FPU in saccharification of *S. lattisima* obtaining near 60% (57g/kg biomass) of glucose after 24h.

On the other hand, literature reports the use of different pretreatments prior to EH of ligno-cellulosic materials such as acid and/or alkaline (Le Tan et al., 2021) physico-chemical (Rhein-Knudsen et al., 2021) and biological pretreatments (Trivedi et al., 2015). Among different biological pretreatments, solid-state fermentation (SSF) with filamentous fungus, such as *Aspergillus awamori*, is a competitive option due to the high capacity of this species to secrete hydrolytic enzymes (Díaz et al., 2009).

This paper studies the production of sugars by the enzymatic hydrolysis of biologically pretreated *D. dichotoma* with *A. awamori*. As far as we know it is the first time that *Dictyota dichotoma* is used as raw material for this purpose. The biological pretreatment was optimized by assaying different fungal growth temperatures (30ºC and 50ºC) and inoculum sizes (10^6; 10^7 and 10^8 spores/g of dried solid) during the SSF process. In addition, three different enzyme dosages were tested for the subsequent hydrolysis step of pretreated biomass (10; 20

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and 112 FPU/g of dried biomass). The best operating conditions were selected to obtain the maximum free sugars production.

2. Materials and methods

The present investigation includes the growth and application of the biological agent *Aspergillus awamori* to pretreat the algae *D. dichotoma.* Afterwards, the pretreated fungal biomass is enzymatically hydrolyzed to obtain an effluent rich in monosaccharides.

2.1 Algae sampling and conditioning

Seaweed used in the present study was collected in February-March 2021 from “Playa del Carmen” beach (Latitude: 36.1861 Longitude: -5.922), in Barbate (Cádiz, Spain). Seaweed was washed to dilute the concentration of salts and to eliminate sand and other contained impurities. For this purpose, they were placed in water cans and washed by continuous addition of tap water whose discharge was done through the overflow. Then, to remove water which speeds up the decay of the algae, and to ensure long-term storage, the fungal biomass was dried at ambient temperature for two days in a greenhouse. Dried algae were finely ground with a kitchen blender and stored until use.

2.2 Biological pretreatment

Biological pretreatment was performed by solid-state fermentation (SSF) with the fungus *Aspergillus awamori.* Conidia production was obtained from culturing *A. awamori* on Petri dishes using LB medium for 5 days at 30ºC. After that, conidia were harvested by washing the plate with 2 mL of sterile water containing 0.9% (w/v) NaCl. The obtained conidia suspension was used as inoculum. The counting was done using a Neubauer Chamber with the help of an optic microscope type Leica DME. SSF was carried out in static conditions in 250 mL Erlenmeyer flasks. Each flask contained 2 g of dried and ground *D. dichotoma* moistened at a 1:3 solid/liquid ratio using the Minimal Medium solution (Marzo et al., 2020). Flasks were autoclaved at 120°C for 20 min to decontaminate the algae feedstock by eliminating endogenous microorganisms. Then, they were cooled and inoculated with conidia suspension. Different conditions were tested: inoculum concentration (10^6; 10^7 and 10^8 spores/g of dried solid) and temperature (30ºC and 50ºC).

2.3 Analysis

The inorganic composition of the seaweed was evaluated by its salinity profile after its lixiviation. For this purpose, 10 g of the seaweed was incubated with 100 mL of distilled for 1h. The anions in the lixiviate were determined by ionic chromatography (Metrohm 930 compact ic flex and Metrohm 940 professional ic vario models). The pretreatment effect on the seaweed was determined by measuring the fibers profile of the biomass before and after the pretreatment using a fiber analyzer (Fibertec 8000-FOSS) following the procedure described by Ricci et al. (2019).

2.4. Hydrolysis

For the hydrolysis, 1g of pretreated macroalgae was mixed with 45 mL phosphate buffer (0.05 mol/L, pH 5) in 250 mL Erlenmeyer flasks and sterilized in an autoclave (121 °C for 20 min). After that, flasks were cooled down and the enzyme Cocktail Cellic® Ctec2 from Novozyme was added. Immediately, flasks were incubated in an orbital shaker (IKA KS 4000i control) at 50 °C and 150 rpm for several days. During the hydrolysis process, samples were taken periodically. They were centrifuged at 11,000 g for 10 min (Eppendorf 5810R) collecting the supernatant for the analysis of total reducing sugars (TRS) by the DNS method adapted to microtiter plate (Gonçalves et al., 2010).

3. Results and Discussion

Firstly, seaweed was characterized in terms of anions and fiber composition. Afterwards, the effect of different conditions of the biological pretreatment on subsequent enzymatic hydrolysis was analysed.

3.1 Characterization of *D. dichotoma*

Results obtained in the inorganic anions and fiber analysis of *D. dichotoma* are shown in Table 1. In the lixiviate fraction, the conductivity was slightly higher than that for tap water (0.5-0.8 mS/cm) due to the presence of different ions. The fiber analysis of non-pretreated algae showed that mucilage content (Xylo-fuco-glucans) accounted for 58% (w/w), 40% of which made up complex mucilages in form of salts, such as sulfated laminarin,

while the remaining 18% was found as free mucilages. Brown macroalgae from the *Phaeophyta* division have a high content of alginates which are normally forming salts of Calcium (Ca); Magnesium (Mg) or Sodium (Na); elements which were also measured in the lixiviate (Utsolsteva et al., 2018). Sugars extracted by acids such as cellulose, fucose or galactose represent 25% (w/w). Diluted acid can extract some sugars such as uronic acids, xylose and mannose; however, other common sugars such as cellulose, fucose or galactose represent 6.59% of dry matter (concentrated acid fraction). It is important to remark the low content of compounds extracted by acetone such as lipids and pigments. Normally chlorophylls, β-carotenoids and fucoxanthin and xanthophylls are present in the case of Division *Phaeophyta* (Rabanal 2015).

Table 1: Inorganic anionic analysis of D. dichotoma

Solid Characterization	Values (%)
Total Solids	86.8
Water	13.2
Inorganic matter	10.9 ± 1.1
Organic matter	75.9 ± 1.1
Acetone extracted fraction	0.4 ± 0.0
Neutral detergent extracted fraction	57.7 ± 1.8
In form of salts	40.4 ± 1.1
Free forms	17.2 ± 0.9
Diluted acid extracted fraction	18.5 ± 1.2
Concentrated acid extracted fraction	6.6 ± 1.4
Organic-No extractable fraction	6.5 ± 0.9
Inorganic salts	10.9 ± 1.1
Total salts	51.6 ± 0.8
Liquid Characterization (Lixiviate)	**Concentration (mg/L)**
Conductivity	1.2 mS/cm*
Fluorides	7.15
Chlorides	44.2
Nitrite	0.211
Bromide	0.435
Nitrate	2.42
Phosphate	5.30
Sulfate	40.7
Sodium	20.4
Ammonium	2.01
Potassium	13.3
Magnesium	9.96
Calcium	46.0

**Conductivity unit: mS/cm*

3.2 Effect of Temperature and Inoculum size of Solid-State Fermentation on Sugar release.

The results obtained after fiber analysis of *D. dichotoma* biologically pretreated at different temperatures are shown in Table 2.

Table 2: Fiber analysis of D. dichotoma after 15 days of SSF at 30ºC y 50ºC

Fractions	SSF-30ºC	SSF- 50ºC
Acetone extracted fraction	1.19	1.51
Neutral detergent extracted fraction	54.1	63.8
In form of salts	21.0	21.6
Free forms	33.1	42.2
Diluted acid extracted fraction	23.2	17.2
Concentrated acid extracted fraction	12.7	12.6
Organic-No extractable fraction	8.83	5.00
Inorganic salts	0.00	0.00
Total salts	21.0	21.6

For pretreated algae at both temperatures assayed, salty mucilages were reduced by about 20%, whereas free mucilages increased by 16% and 25% for 30ºC and 50ºC, respectively. This fact can be explained taking into account that cellulases from *A. awamori* are more active at 50ºC than at 30ºC (Liu et al., 2020). So the cellulosic structures of seaweed, such as laminarin, could be more efficiently hydrolyzed at 50ºC as a consequence of the secretion of enzymes needed for the fungus growth during the biological pretreatment, releasing the most available monomers such as glucose and mannitol. As a consequence of this aspect, other carbohydrates such as fucoidan may become more accessible to fungal attack. This was reflected in the increase of the acid-extracted fraction, which rose by 6% after biological pretreatment at both temperatures. However, at 30ºC the acid-diluted fraction, which contains uronic acids among other fucoidans, was 1.4-times higher, probably due to the activation of other fungal enzymes such as alginate lyase, which shows maximum activity at 37ºC. (Singh et al., 2011). In addition, the deactivation of the enzymes secreted by A. awamori is low at 30ºC (Malikoglu et al., 2013). Thus, the net increase in total sugars (sugars extracted in the fractions with dilute and concentrated acid) was 10% and 5% for 30ºC and 50ºC, respectively. Hence, 30ºC was selected as the optimal temperature for subsequent studies. Pretreated seaweed at 30ºC with three different inoculum sizes was hydrolyzed for 24 h with Cellic® Ctec2. Reducing sugars measured for biologically pretreated and non-pretreated *D. dichotoma* are shown in Figure 1.

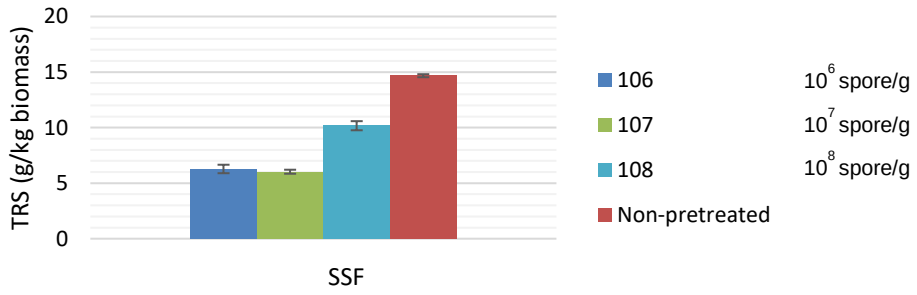


Figure 1: Total Reducing Sugars (TRS) from biologically pretreated and non-pretreated seaweed. Pretreatment conditions: Biological agent: A. awamori; Solid-liquid ratio: 1:3 w/v; T = 30ºC; t =15 d. EH conditions: Dosage of enzyme: 10 FPU/g biomass; t = 24h; pH = 5, T= 50ºC.

Among the inoculum sizes tested, 10^8 spores/g of dried solid allowed to reach the maximum TRS (10.2 g/kg biomass). However, the EH of non-pretreated seaweed gave a slightly higher concentration (14.7 g/kg biomass). The use of lower concentrated inoculums reduced, even more, the production of TRS (Figure 1). Therefore, it seems that the biological pretreatment at the conditions assayed is not adequate to pretreat fungal biomass. Some authors which have obtained positive results with the fungal biological pretreatment of macroalga have used Mendel's solution to adjust the initial moisture; perhaps this can improve the SSF (Yahmed et al., 2017).

3.3 Effect of enzyme dosage on Sugar release.

Three different enzyme dosages (10, 20 and 112 FPU per g of biomass) were tested to hydrolyze the seaweed. Those experiments were performed with non-pretreated *D. dichotoma* (Figure 2).

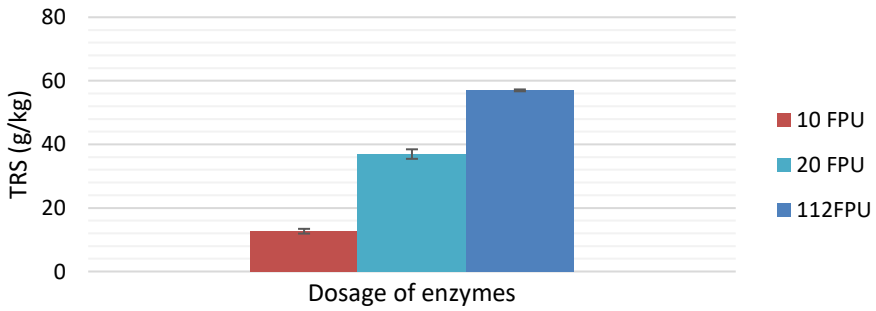
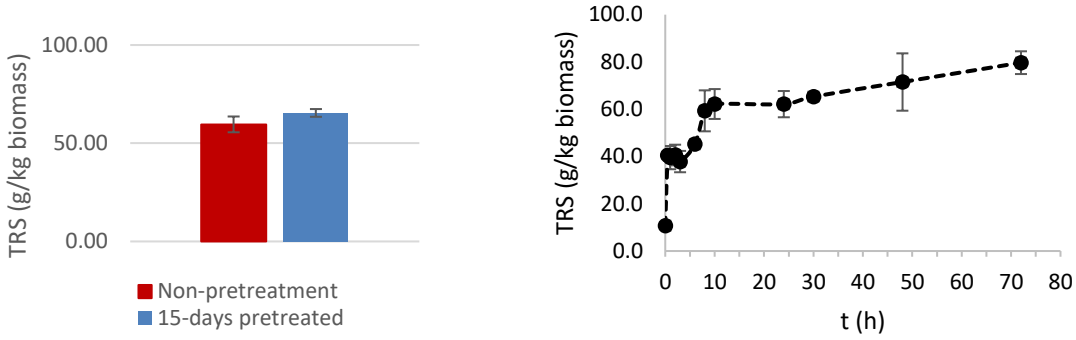


Figure 2: Total Reducing Sugars (TRS) after 24h of enzymatic hydrolysis using Cellic® Ctec2. Pretreatment conditions: Biological agent: 10^8 cel of A. awamori /g dried solid; Solid-liquid ratio: 1:3 w/v; T = 30ºC; t =15 d. EH conditions: t = 24h; pH = 5, T= 50ºC.

The maximum reducing sugars concentration measured in the hydrolysates was 57 g/kg of biomass when 112 FPU/g biomass were added. Other authors have used other hydrolytic enzymes to supplement cellulase for the saccharification of brown seaweed. For example, Saravanan et al., (2018) used cellulase (53 FPU/g biomass) plus pectinase (20 U/g biomass) for EH of the brown seaweed *Sargassum sp*. They obtained 60 g of TRS per kg for acid pretreated biomass and increased up to 110 g/kg when acid pretreated seaweed was subsequent enzymatically hydrolyzed with cellulase plus pectinase.

3.4. Sugar release from enzymatic hydrolysis of biologically pretreated seaweed

So, a complex process is proposed for sugar production from *D. dichotoma* at the following conditions: biological pretreatment by solid-state fermentation with *A. awamori* for 15 days at 30ºC using an inoculum concentration of 10^8 spores/g, followed by enzymatic hydrolysis with 112 FPU/g biomass of Cellic® Ctec2 at pH 5, 50ºC and 150 rpm for 72h. The total reducing sugars achieved after 15 days of pretreatment (Figure 3.A) and the temporal evolution of RS along the hydrolysis of pretreated seaweed for 15 days (Figure 3.B) are shown below.



(A) (B)

Figure 3. A. Total Reducing Sugars (TRS) reached after 24h of EH of biologically pretreated and non-pretreated seaweed. B. Temporal evolution of TRS from 15-days pretreated seaweed. Pretreatment conditions: Biological agent: 10^8 spores of A. awamori /g dried solid; Solid-liquid ratio: 1:3 w/v; T = 30ºC; t =15 d. EH conditions: Dosage of enzyme: 112 FPU/g biomass; pH = 5, T= 50 ºC.

As it can be seen in Figure 3.A, TRS concentration from 15-days pretreated seaweed is 10% TRS higher than that for non-pretreated biomass. Figure 3.B shows the temporal production of TRS produced along the EH of biologically pretreated sample. It can be seen that a sharp increase is produced in the first 24 h and from 24 to 30 h, around 65 g/kg biomass was reached, although the highest value was measured after 72 h of hydrolysis (80 g/Kg). However, an economic evaluation should be done to asses if it is worth increasing the time of EH from 24 h. In this respect, other authors have obtained 300 g/kg biomass with much lower doses of enzymes (Sharma and Horn, 2016). However, those authors used a solid/liquid ratio for the EH 2.3 times higher than the one used in our study. Also, Azizi et al., (2017) obtained 17 times higher TRS than in this study by using 5 times higher solid-liquid ratio (10% w/v) and less amount of enzymes (15 FPU of cellulase and 15 IU of cellobiase /g biomass). Therefore, new investigations reducing the dosage of enzymes and increasing the solid-liquid ratio are needed to optimize *D. dichotoma* sugar release.

4. Conclusions

The release of sugars of macroalgae *D. dichotoma* has been performed by the application of a process consisting of a biological pretreatment with the fungus *A. awamori* for 15 days followed by an enzymatic hydrolysis stage. From the two temperatures studied for the pretreatment, 30ºC and 50ºC, the lowest was selected because it favors the solubilization of the diluted acid extracted fraction of the macroalgae. Among the inoculum sizes assayed, 10^8 cel/g of dried biomass was chosen because allowed a higher TRS release after the hydrolysis of the fermented seaweed. At these conditions, the EH for 24 hours of pretreated seaweed gave a 31% TRS release higher than for non-pretreated biomass. After EH for 72h, a value as high as 80 g of TRS/kg biomass was measured. This process could be further optimized by the increasing solid-liquid ratio for the hydrolysis stage and reducing the dosage of enzymes to obtain an effluent rich in sugars that can be used for any other process.

Nomenclature

TRS – total release of sugars, g/L or g/kg biomass
T – temperature, ºC
FPU – filter paper units, -
t – time, h

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