SOLID-STATE FERMENTATION OF *ULVA RIGIDA* FOR PRODUCTION OF CELLULASES, XYLANASES AND B-GLUCOSIDASE

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Abstract

Seaweeds are important organisms in marine ecosystems, are included on blue biorefinery category and they are rich in different polysaccharides, that can induce enzyme production by solid-state fermentation (SSF). *Ulva rigida* was used as substrate in SSF to produce cellulases and xylanases and to increase its protein content. The SSF were performed with previously washed and with unwashed seaweed by *Aspergillus ibericus*. Xylanase and cellulase activities were higher in fermented unwashed seaweed, correspondingly 359.8±6.1 U/g dry substrate and 59.9±2.4 U/g, respectively. In fermented washed *U. rigida*, these values achieved 212.4±17.7 U/g and 43.6±3.7 U/g, respectively. β-glucosidase activity was similar in washed seaweed (6.94±0.21 U/g) and in unwashed seaweed (6.87±0.18 U/g). After SSF, protein content of unwashed seaweed was increased by 14%. Unwashed *U. rigida* resulted in better enzymatic activities, being a promising option to produce these commercially-valued enzymes.

INTRODUCTION

Green seaweeds, as *Ulva rigida*, have higher quantities of cellulose in contrast with brown and red macroalgae which makes them an ideal substrate for bioenergy production (Karray *et al.*, 2016). Algae biomass is included in the blue biorefinery concept (Abdul Manan & Webb, 2017) and can be incorporated in the bioeconomic cycle to reduce our

dependency of limited fossil energy sources, combining the potential of biorefineries to process algal biomass into different products and biofuels (Laurens et al., 2017).

Non-starch polysaccharide (NSP) enzymes are gaining more attention and represent a high-value income due its potential in the production of bioethanol of second generation and as additive in monogastric animals feed formulation (Adeola & Cowieson, 2011; Castillo & Gatlin, 2015).

Solid-State Fermentation (SSF) is a biotechnological process that recreates microbial natural conditions of growth, specifically of filamentous fungi (Abdul Manan & Webb, 2017). One of the filamentous fungi species used in SSF is *Aspergillus ibericus*, "generally regarded as safe" (GRAS) (Oliveira *et al.*, 2016), whose hyphal growth mode permits to penetrate in the substrate more efficiently.

In the present work, the production of valuable carbohydrase enzymes, such xylanases, cellulases and β-glucosidase by SSF of washed and unwashed *U. rigida* were evaluated. After SSF, changes in nutritional composition of seaweeds were studied.

METHODOLOGY

Ulva rigida was provided by *Algaplus*, a company based in Aveiro, Portugal. Dry micronized *U.* rigida was used as is or washed, by immersion in distilled water (1:5 w/v) for 48 hours, under constant agitation (150 rpm), filtrated by vacuum and dried at 55 ˚C until constant weight.

Solid-State Fermentation (SSF)

SSF was performed in duplicate in Erlenmeyer flasks of 500 mL with 10g of dry unwashed or washed *U. rigida.* Moisture level was adjusted to 75% followed by sterilization at 121 ˚C for 15 minutes. The medium was inoculated with *Aspergillus ibericus* following the method described by Sousa *et al.* (2018) and incubated at 25 ˚C for 7 days. The proximate composition of both unwashed or washed *U. rigida* was determined before and after SSF, and the enzymatic activity after SSF. Fermentation were performed in duplicate.

Seaweed chemical composition

Total protein content (N x 6.25) was determined by Kjedahl method after digestion with Sulphur acid (>95%) using a *Kjeltec* system (Foss 8400). Moisture was analyzed by drying

samples at 105 ˚C until constant weight. Salts' content after wash the seaweed with distilled water for 24h, followed by slow evaporation at 55 ˚C for 24h and incineration in a muffle furnace at 505 ˚C for 2h. Free reducing sugars were determined by DNS (3,5-dinitrosalicylic acid) method. Cellulose and hemicellulose was determined by the method described by Leite et al. (2016).

Enzymatic extraction and analysis

After the fermentation period, the enzymes were extracted following the method of Salgado *et al.* (2014). Two different methods were carried out to assess cellulase activities. In method 1, a commercial kit (S-ACMCL; Megazyme International, Ireland) was used, that included *endo*-1,4-β-glucanase and AZO-carboxymethyl cellulose as substrate and the reaction was performed at 40 ˚C. For method 2, carboxymethyl cellulose (2%) in citrate buffer 0.05N (pH 4.8) was used as substrate, the reaction was performed at 50 ºC for 30 min, and free sugars were determined by DNS method. In both procedures, one unit of enzyme activity was defined as the quantity of enzyme necessary to release 1 µmol of glucose from substrate each minute at the reaction conditions.

Similarly, two methods were used for xylanase activities. In method 1, a commercial kit (Azo Wheat arabinoxylan S-AWAXL 05/14; Megazyme International, Ireland) was used including a specific *endo*-1,4-β-xylanase and the reaction temperature was 40 ºC. In method 2, it was used xylan (1%) in citrate buffer 0.05N (pH 4.8), the reaction was incubated at 50 ˚C for 15 minutes, and the released sugars were measured by DNS method. In both methods, one unit of enzyme activity was defined as the quantity of enzyme necessary to release 1 µmol of xylose reducing sugar equivalents from substrate each minute at the reaction conditions.

β-glucosidase activity was measured following the method described by Leite *et al.* (2016) and its activity was defined as the amount of enzyme required to release 1µmol of p-nitrofenol per minute. All activities were expressed per g of dry substrate in SSF.

All data was analyzed by one-way analysis of variance (ANOVA) using the *Statgraphics Centurion* software. If significant differences were detected (p<0.05) the Tukey multiple range test was used to discriminte means.

RESULTS AND DISCUSSION

SSF was successfully performed in both washed and unwashed *U. rigida*. Protein and polysaccharides content of unwashed and washed *U. rigida* are presented in Table1. During SSF of washed seaweed loss 6.7% of the initial biomass and unwashed seaweed loss 12.7%. Protein content of unwashed seaweed increased circa 10.2% of protein after SSF. In both unwashed and washed *U. rigida*, reducing sugars decreased in after SSF. This reduction was probably due to the fungi consumption of this carbon source which is important to promote its growth in the substrate (Farinas, 2015). The cellulose and hemicellulose fraction were reduced after SSF of unwashed seaweed 61.3 % and 33.6 % respectively, and they were transformed to fungal protein. Thus, their nutritional value was increased to be used in monogastric animal feed.

Table 1. Composition of unwashed and washed *U. rigida* before and after SSF with *A. ibericus*.

	Unwashed seaweed		**Washed seaweed**	
	Before SSF	*After SSF*	*Before SSF*	*After SSF*
Protein (g kg^{-1})	169.1 ± 0.66 ^{a}	212.5 ± 2.6 ^{b}	235.5 ± 1.30 ^{c}	256.7 ± 4.29 ^{d}
Reducing sugars (mg g^{-1})	15.72 ± 0.33 ^{a}	9.28 ± 1.72 ^{b}	6.87 ± 0.29 ^{c}	2.96 ± 0.18 ^{d}
Celullose (%)	9.53 ± 1.39 ^{a}	4.23 ± 0.01 ^{b}	14.0 ± 0.81 ^{a}	4.39 ± 0.13 ^{b}
Hemicelullose (%)	11.72 ± 0.77 ^{a}	8.92 ± 0.01 ^{b}	12.28 ± 0.19 ^{a}	12.51 ± 0.43 ^{a}

Values presented as mean ± standard deviation. Means with different letters are significantly different ($p<0.05$)

Enzymatic activity of washed and unwashed *U. rigida* after SSF is presented Table 2. Higher xylanase and cellulase activities were found in fermented unwashed seaweed, using both analytical methods, while β-glucosidase activity was not statistically affected.

Differences in enzymatic activities between washed and unwashed seaweed is probably related with the higher quantity of reducing sugars present in unwashed compared to washed *U. rigida*. Reducing sugars probably stimulated fungi growth, producing higher quantity of enzymes (Abdul Manan & Webb, 2017) and so resulting in better substrate utilization. Also, the washing process removed 225.4 g kg^{-1} of salts, which may indicate the wash out of some vestigial elements such as minerals, probably impoverishing the substrate nutritional conditions to support fungi optimal growth (Farinas, 2015).

The high cellulase and xylanase activities of fermented *U. rigida* have high application potential as feed additive for terrestrial monogastric and aquatic animals, to promote growth and feed utilization efficiency and to reduce nutrient excretion (Adeola & Cowieson, 2011; Castillo & Gatlin, 2015).

Table 2. Enzymatic activity of washed and unwashed *U. rigida* after SSF with *A. ibericus*.

	Unwashed seaweed		***Washed seaweed***	
Enzyme activities (U/g)	*Method 1*	*Method 2*	*Method 1*	*Method 2*
Cellulase	42.4 ± 3.6 ^{b}	59.9 ± 2.4 ^{c}	19.0 ± 1.6 ^{a}	43.6 ± 3.7 ^{b}
Xylanase	51.8 ± 2.2 ^{a}	359.8 ± 6.1 ^{c}	13.5 ± 0.7 ^{a}	212.4 ± 17.7 ^{b}
β-glucosidase	6.9 ± 0.2 ^{a}		6.9 ± 0.2 ^{a}	

Values presented as mean ± standard deviation. Means with different letters are significantly different ($p<0.05$)
Method 1: Commercial kit (Megazyme International, Ireland)
Method 2: Free sugars determined by DNS

CONCLUSIONS

According to the results obtained, solid-state fermentation was a viable biotechnological tool that can be successfully applied to obtain high-valued compounds, as the non-starch polysaccharides enzymes. In addition, SSF of seaweeds can increase their protein content and reduce the polysaccharides. Further research should rely on the application of these enzymes and fermented seaweeds in animal nutrition, specifically in aquaculture fish species.

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