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Performance of an enzymatic extract in *Botrycoccus braunii* cell wall disruption

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Microalgae can produce and contain lipids, proteins and carbohydrates, which can be extracted and marketed as potential novel added-value bio-products. However, microalgae cell wall disruption is one of the most important challenges involved while processing this type of biomass. In this context, white-rot fungi, responsible for the biodegradation of lignin present in wood due to non-specific extracellular enzymes, could be applied for promoting microalgae cell wall degradation. Therefore, the aim of this study was to evaluate the use of an enzymatic extract produced by the white-rot fungi *Anthracophyllum discolor* as a biotechnological tool for *Botryococcus braunii* cell wall disruption. The fungus was inoculated in wheat grains and manganese peroxidase (MnP) activity was monitored while obtaining the enzymatic extract. Then, cell wall disruption trials with different MnP activity were evaluated by the biochemical methane potential (BMP). In relation to cell wall disruption, it was observed that the optimal value was obtained with enzymatic concentration of 1000 U/L with a BMP of 521 mL CH_4/g VS. Under these conditions almost 90% of biomass biodegradability was observed, increasing in 62% compared to the microalgae without treatment. Therefore, the results indicate that enzymes secreted by *A. discolor* promoted the attack of the different cell wall components finally weakening it. Therefore, the application of this treatment could be a promissory biotechnological approach to decrease the energetic input required for the cell wall disruption step.

[**Key words :** Cell wall disruption; Anthracophyllum discolor; Microalgae; Ligninolytic enzymes; Biochemical methane potential]

Microalgae can contain high amounts of lipids, proteins and carbohydrates, which can be extracted and marketed as potential novel added-value bio-products (1). However, the industry of high added-value bio-products and biofuels produced from microalgae has several drawbacks, such as the use of pure strains and the use of both, expensive culture and harvesting methods. Additionally, the downstream processing represent other of the bottlenecks (2), where the high water content after biomass harvest and microalgae cell wall disruption for extracting these novel bio-products represent the most important energy demanding steps involved in biomass processing (2).

According to Lee et al. (3), cell disruption is particularly important, as the contents of the extracted lipids or other compounds are determined according to the disruption method and device applied. Therefore, the appropriate cell disruption method and device is a key issue to increase the lipids extraction efficiency (3). Disruption methods have been widely used to improve availability of internal compounds such as carotenoids (4), enzymes (5) or lipids (3) from microorganism such as bacteria, yeast and microalgae. These disruption methods can be divided into four main categories (6): mechanical, physical, chemical and enzymatic processes, being the most commonly used methods for microalgae cell wall disruption high-pressure homogenization, agitation with glass or ceramic beads in bead-mills, ultrasonication of suspended microalgal cells, acid or alkaline treatment, cryogenic grinding, microwaves (7) and thermal treatment (8). Current microalgae cell wall disruption processes have a high specific energy consumption, and the energy available in the extracted lipids cannot even meet the energy requirements for cell disruption (9). Additionally, currently available cell disruption techniques focused on one specific product cause complete disruption of the cells damaging the other valuable components present in the microalgae (1). In the case of the microalgae biorefinery process, it is necessary the search for low energy demanding and selective methods for cell disruption to obtain different products from the cell.

The cell wall present in most of the microalgae species, is typically constituted by tri-layered structures which include polysaccharides such as cellulose, uronic acid, protein, mannose, xylan

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or trilaminar layers of algaenan; glycoproteins; and minerals such as calcium or silicates (9). These materials form tough cell wall structures with a tensile strength of about 9.5 MPa. This value is about the same as bacteria or yeast but three times higher than that of plant cells (9). Therefore, depending on cellulose content the algal cell wall could be treated as any other cellulosic ethanol feedstock as algal cellulose is similar to plant cellulose (10). There are only few works related with the application of enzymatic treatment for microalgae cell wall disruption, such as autolysis (11), the use of enzyme mixtures (12) or sole enzyme treatment (13). However, the majority of the works use commercial enzymes, increasing the pretreatment costs.

The capacity of some white-rot fungi to degrade lignocellulosic materials is well known. *Anthracophyllum discolor* (*Xerotus discolor*), a white-rot fungus isolated from the forest of southern Chile, has demonstrated a high production of ligninolytic enzymes such as manganese peroxidase (MnP), which have shown relevant biodegradative properties (14). Therefore, the enzymes produced by *A. discolor* could be applied as a biotechnological alternative to physico-chemical methods for cell wall disruption of the microalgae *Botryococcus braunii*. In this sense, the aim of this work was to evaluate the use of an enzymatic extract of *A. discolor* for disrupting the cell wall of *B. braunii*.

MATERIALS AND METHODS

Strain and culture The green microalga *B. braunii* LB 572 race B from UTEX was selected for all experiments. This strain grows in colonies and was produced in panel reactors made of polyethylene according to Bazaes et al. (15). The microalgae were harvested using a continuous centrifuge, obtaining a paste with a 20% (wt) of dry matter. Then, the paste was dried at 40 °C for 24 h, reaching a 90% (wt) of dry matter.

A white-rot fungus *A. discolor*, isolated from decayed wood in the rain forest of southern Chile, was used in this study (14). The fungus was transferred from slant tubes (maintained at 4 °C) to glucose malt extract agar plates (15 g/L agar, 3.5 g/L malt extract, and 10 g/L glucose) and kept at 30 °C for 5–7 days before its use.

Enzymatic extract of *A. discolor* One hundred mL of modified Kirk medium (16) were placed in 2 L Erlenmeyer flasks and then inoculated with five malt agar plugs (6 mm diameter) of active mycelium of *A. discolor*. The fungal culture was incubated at 25 °C and after 7 days was homogenized in a sterilized blender for 1 min. The blended mycelia were used as the inoculum. Then, 2 g of wheat grains and 6 mL of distilled water were added to a 100 mL Erlenmeyer flask and autoclaved at 121 °C and 1 atm for 40 min twice consecutively.4 mL inoculum were added into the flasks and the cultures were incubated at 25 °C for 28 days. The MnP activity was periodically monitored in the liquid culture in order to identify the day of higher enzyme production. Finally, in the day of high enzyme production the mycelium was separated from the culture medium by filtration using a Whatman membrane filter (0.45 μm). The filtrate was concentrated by ultrafiltration using Amicon tubes (10 kDa) at 4 °C for obtaining the final enzymatic extract.

Biotechnological cell wall disruption assays For these assays a concentrate enzymatic extract of 7000 U/L was used in different dilutions to obtain the following enzymatic activities: 7000; 4000; 2000; 1000 and 500 U/L, respectively. For the reaction, 1 mL of each solution were added to 0.5 g of dried microalgae biomass [90% (wt)] in glass vials and incubated at room temperature for 24 h and 200 rpm in an orbital shaker.

The biodegradability of the cell wall could be associated to the disruption degree (17). Therefore, biochemical methane potential (BMP) assays were performed. The BMP was determined through pressure increase in the headspace of experiments developed in 117 mL serum bottles, using 50 mL of medium (18). The medium for methanogenic bacteria was composed of yeast extract, macronutrients and bicarbonate. After the enzymatic treatment, the samples were digested by the anaerobic consortia at 30 °C in serum bottles. Treated microalgae (substrate) and anaerobic sludge were added at a 1:1 ratio (g/g). Biodegradability of the samples was evaluated in terms of BMP, which indicates methane produced due to microalgae digestion. The BMP was expressed as methane volume produced per gram of microalgae (mL CH_4/g VS). Also, the quality of biogas produced was measured through gas chromatography coupled with a thermal conductivity detector (TCD). Biodegradability efficiency was estimated through comparison between theoretical and real BMP.

Additionally, observations in a Confocal Laser Microscopy Olympus FluoView 1000 were performed providing qualitative information about microalgae cell wall lysis. The samples were observed using an objective lens of 40×, with 3 lasers of 405 nm, 488 nm and 635 nm, respectively.

Analytical methods MnP activity was measured from the supernatant of a previously centrifuged sample (10 min at 5000 rpm). MnP activity was determined by spectrophotometrically monitoring the oxidation of 2,6-dimethoxyphenol (2,6-DMP) at 30 °C (Cecil CE 7200, Cecil Instruments, UK). The reaction mixture (1 mL) contained 200 mL (250 mM, pH 4.5) of sodium malonate, 50 mL (20 mM) of 2,6-DMP, 50 mL (20 mM) of $MnSO_4 \cdot H_2O$, and 600 mL of supernatant (10 min at 5000 rpm). The reaction was initiated by adding 100 mL (4 mM) of H_2O_2. The absorbance of the colored product was measured at 468 nm and corrected for lacasse (Lac) activity (19). One MnP activity unit (U/L) was defined as the amount of enzyme transforming 1 mmol of 2,6-DMP per minute.

RESULTS AND DISCUSSION

Enzymatic extract of *A. discolor* MnP activity was evaluated to identify the day of maximum enzyme production by *A. discolor* cultivated in a media containing wheat grains, for further recovery of the enzymatic extract (Fig. 1). The maximum MnP activity was detected after 19 days from the inoculation, reaching a value of 645 U/L. The result is in agreement with studies developed by Acevedo et al. (20) and Rubilar et al. (21) in which high MnP activity has been detected when *A. discolor* is incubated in a media containing wheat grains. During the day of maximum MnP activity, the enzymatic extract was collected and concentrated by ultrafiltration with Amicon tubes (10 kDa). The enzymatic extract obtained which presented an MnP activity after ultrafiltration of 7000 U/L was stored for further studies.

Biotechnological cell wall disruption assays Several parameters may determine if a method is sustainable or not for microalgae cell wall disruption. According to Lee et al. (9) parameters like toughness of the cell wall, the economics of the process and the ease of scalability from laboratory to industrial scale are the most relevant ones. In the case of biofuels production, the method applied must be economically feasible because biofuels are commodities with a low market price. However, in the case of the obtention of high added-value bioproducts or a biorefinery process, where multiple compounds are produced, the disruption methods applied must not cause a damage. Therefore, the ability of the extracellular enzyme extract produced by *A. discolor* for the degradation of *B. braunii* cell wall, was tested applying different dilutions of the concentrated enzymatic extract in terms of MnP activity. According to Alzate et al. (17), the BMP assay constitutes a useful tool to determine both the ultimate biodegradability and the methane production yield of organic substrates. However, the recalcitrant compounds found in the cell walls of microalgae showed a low biodegradability, which make them highly resistant to bacterial

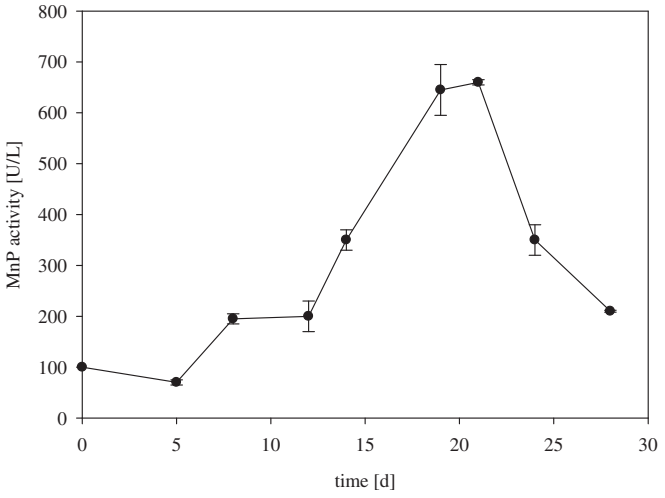

FIG. 1. MnP activity of A*nthracophyllum discolor* cultivated with wheat grains.