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Saccharification of *Spirulina platensis* biomass using free and immobilized amylolytic enzymes

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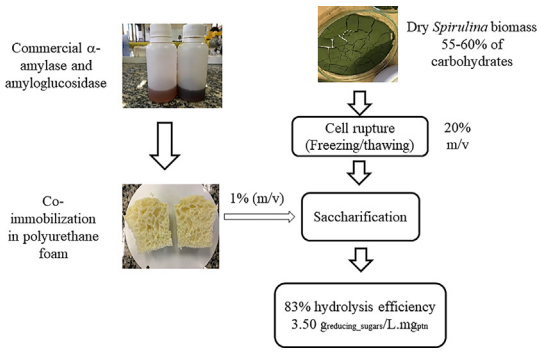
GRAPHICAL ABSTRACT



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ABSTRACT

We aimed to use physical methods of microalgal biomass rupture to study saccharification strategies using free and immobilized amylolytic enzymes. The biomass of *Spirulina platensis*, which consists of 50–60% carbohydrates, was exposed to physical cell rupture treatments, with better results obtained using freeze/thaw cycles following by gelatinization. In saccharification tests, it was possible to hydrolyze *Spirulina* biomass with hydrolysis efficiencies above 99% and 83%, respectively, using 1% (v/v) of free enzymes or 1% (m/v) of amylolytic enzymes immobilized together. The use of free and immobilized enzymes yielded high levels of conversion of polysaccharides to simple sugars in *Spirulina* biomass, showing that these processes are promising for the advancement of bioethanol production using microalgal biomass.

1. Introduction

Biofuels obtained from various types of biomass have been proposed as an alternative to fossil fuels, which although widely used, have been recognized as causing numerous environmental problems (Pejin et al., 2015; Ullah et al., 2015). Algae and microalgae are among the biomasses with potential for use (Hallenbeck et al., 2016; Novoveská et al., 2016) and present advantages over other biofuel sources (sugarcane, corn, canola, soybean) as they are directly linked to food production for arable areas (Su et al., 2017).

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Microalgae produce higher yields in biofuels (24,355–136,886 L/ha·a^{-1}) than agricultural crops such as soybean (1,235 L/ha·a^{-1}) and maize (3,571 L/ha·year^{-1}) (Singh et al., 2011). In addition, they can be grown in alternative media such as effluents and agroindustrial residues (Salla et al., 2016; Usha et al 2016; Canter et al., 2015).

The type of biofuel produced depends on the carbohydrate, lipid, and protein content of the microalgal biomass (Tercero et al., 2014). For bioethanol production, the intracellular carbohydrates of microalgae are used (Chen et al., 2011). According to Usharani et al. (2012), the reserve product of *Spirulina* is a polysaccharide formed by glucose monomers linked by α-1,4-type glycosidic linkages, known as cyanophyte starch. This polysaccharide differs from the starch because it has more abundant branches in relation to the main chain of polysaccharide. For the hydrolysis of the starch of cyanophytes, microalgal cell wall breakdown and polysaccharide hydrolysis are necessary steps prior to the fermentation processes (Baeyens et al., 2015; Hernández et al., 2015).

Enzymatic hydrolysis is preferred over acid hydrolysis, which generates inhibitory byproducts (Pancha et al., 2016). In addition, enzymatic hydrolysis can be carried out under mild reaction conditions to yield products with enhanced purity (Juturu and Wu, 2014). However, disadvantages include the high cost of biocatalysts as well as possible inactivation due to process conditions (Choi et al., 2010).

Depending on the type of carbohydrate matrix present in the cell wall, non-fermentable sugars, such as pentoses, can be generated by yeast during fermentation, and it is important to use biomasses that do not generate large amounts of these products during hydrolysis (Abdullah et al., 2014). The *Spirulina* cell wall contains polysaccharides predominantly composed of hexoses, with low levels of xyloses and other pentoses, making it a promising biomass for the production of bioethanol (Blinkova et al., 2001).

Enzyme immobilization technologies allow for the reduction of process costs by increasing the specific activity of the enzymes, reducing the likelihood of inactivation by pH and temperature changes, and allowing for the separation and reuse of the enzymes (Vaz et al., 2016; Rodrigues et al., 2017; Cunha et al., 2014). Few studies have used immobilized enzymes for the hydrolysis of algal biomass, and only one has reported using the polyurethane foam immobilization method described in our work (Rodrigues et al., 2017).

Few studies have been carried out with the subject of enzymatic saccharification of *Spirulina* polysaccharides in the last years (Rodrigues et al., 2017; El-Mashad, 2015; Chen et al., 2013) demonstrating the scarcity of studies in this area. Our study aims to deepen current knowledge about these new sources of bioethanol production.

We investigated pretreatment methods that promoted more efficient activity of commercial amylolytic enzymes on *Spirulina* biomass. In addition, we characterized the optimal temperatures and pHs at which both enzymes would exhibit similar activities. The enzymes were immobilized and used in saccharification of the biomass, and the results from this method were compared with those obtained when using free enzymes.

2. Material and methods

2.1. Microalgae biomass production and characterization

The cyanobacteria *Spirulina platensis* LEB-52 was cultivated in mini raceways constructed with acrylic, 1.00 m in length and 0.20 m in width, as proposed by Magro et al. (2017). Each raceway has a useful volume of 10 L and is equipped with a paddle wheel, where the stirring speed is controlled by the rotation of the blades. The mini raceways were placed in a greenhouse of transparent film at a controlled temperature (21–35 °C) and received ambient light that was not regulated. The initial agitation speed used was 0.35 m/s.

When the cyanobacteria reached the stationary phase of growth, the culture was ended. Separation of the biomass was performed by filtration over a nylon mesh of 0.048 mm wires with subsequent oven drying at 50 °C for 24 h. The dried biomass was characterized chemically.

Sonication of the dry biomass for cell disruption was performed to prepare samples for quantification of carbohydrate and protein levels. For this, 5 mg of biomass was added to 10 mL of distilled water and then sonicated for five cycles of 59 s in a cell disruptor device (Unique Group, model De S500 11, Indaiatuba-SP, Brazil). The carbohydrate content was determined by the phenol-sulfuric method (Dubois et al., 1956) and protein content was determined according to Lowry et al. (1951). The lipid concentration of the cyanobacteria biomass was determined by the method of Colla et al. (2004). Moisture and ash content were determined by the AOAC Official Methods of Analysis (2000). The carbohydrate, protein, and lipid content were presented on a dry basis.

2.2. Enzymes

The enzymes used in our work were α-amylase (Liquozyme® Supra 2.2X) and amyloglucosidase (AMG® 300 L), which were donated by Novozymes. These are industrial enzymes with the following characteristics: Liquozyme® Supra 2.2X obtained from *Bacillus licheniformis* with declared activity of 300 KNU/g; AMG® 300 L obtained from a selected strain of *Aspergillus niger*, with declared activity 300 AGU/mL.

2.3. Microalgal biomass cell rupture

Cell rupture procedures were used to achieve cell wall disruption of microalgae and release intracellular polysaccharides. Several different physical methods were used (Table 1). For each treatment, 10 g of dried biomass were diluted in 100 mL of sodium phosphate buffer (0.2 M, pH 5.5) (10% w/v), in a 250 mL Erlenmeyer flask. All tests were conducted in triplicate.

Following cell disruption, enzymatic saccharification was performed to evaluate which method of cell disruption was more efficient for the release of carbohydrates. Saccharification was performed by the addition of α-amylase and amyloglucosidase. Both enzymes (1 mL of each one) diluted in distilled water at a ratio of 1:200 (v/v) were added simultaneously to each 100 mL suspension of pretreatment tests, followed by incubation at 50 °C in a shaker with an agitation speed of 150 rpm. Samples were taken at 1 h and 2 h after enzyme addition, at which time the reducing sugars were determined.

Table 1
Experimental design of tests of cell rupture of microalgal biomass.

Test	Method	Description of method
1	Ultrasound*	A suspension of *Spirulina* 10% in 0.2 mol/L sodium phosphate buffer, pH 5.5, was sonicated for 10 cycles of 60 s.
2	Gelatinization	A suspension of *Spirulina* 10% in 0.2 mol/L sodium phosphate buffer, pH 5.5, was gelatinized in a thermostatic bath at 100 °C for 10 min.
3	Autoclaving	A suspension of *Spirulina* 10% in 0.2 mol/L sodium phosphate buffer, pH 5.5, was autoclaved at 121 °C for 20 min.
4	Freezing/Thawing*	A suspension of *Spirulina* 10% in 0.2 mol/L sodium phosphate buffer, pH 5.5, was frozen for 24 h and then thawed.

* Methods followed gelatinization in thermostatic bath at 100 °C for 10 min.