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Apple pomace as a substrate for fungal chitosan production in an airlift bioreactor

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ABSTRACT

The aim of this work was to evaluate apple pomace as carbon source for fungal chitosan production in an external-loop airlift bioreactor by *Gongronella butleri*. Apple pomace has high moisture content, insoluble carbohydrates such as cellulose, hemicellulose and lignin, reducing sugars like glucose and fructose and other sugars such as sucrose. Due to its high content of sugars, apple pomace can be a potential substrate for fungal chitosan production. To improve chitosan production from apple pomace, the effect of the nitrogen sources $NaNO_3$ and $(NH_4)_2SO_4$ and specific aeration rate of 0.1, 0.3 and 0.6 vvm was investigated. The ammonium sulfate and specific aeration rate of 0.6 vvm had significant effect ($p < 0.05$) and showed higher degree of deacetylation and chitosan concentration in the *G. butleri* biomass. Apple pomace seems to be a potential substrate for fungal chitosan production by *G. butleri* and its use can reduce the production cost of this biopolymer and reduce the environmental impact of this agro-industrial waste.

1. Introduction

Chitosan is a cationic biopolymer consisting of β(1→4) bonds between 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose, derived from the deacetylation of chitin (β(1→4)-N-acetyl-D-glucosamine), the second major polysaccharide found in nature.

It has various applications such as in the food (Hosseini et al., 2013; Ma et al., 2013; Peng and Li, 2014), pharmaceutical (Zamani and Taherzadeh, 2012; Qu et al., 2013; Justin and Chen, 2014) and chemical and textile industries (Qin et al., 2006; Travlou et al., 2013), as well as in dental treatment and manufacture of artificial skin (Niederhofer and Müller, 2004; Muzzarelli, 2009; Croisier and Jérôme, 2013) in addition to health benefits such as reducing the absorption of lipids (Neyrinck et al., 2009).

Chitosan can be commercially obtained from partial deacetylation of chitin found in the exoskeleton of crustaceans (Kumar, 2000; Niederhofer and Müller, 2004), although it has disadvantages such as the use of concentrated alkaline solutions at high temperatures, seasonality of the raw material, product with heterogeneous characteristics and low degree of deacetylation (Tajdini et al., 2010). An alternative to chitosan obtained from crustaceans is the production of fungal chitosan by the use of microorganisms capable of producing chitosan in their cell wall such as zygomycetes, where chitin is synthesized by the action of chitin synthase and stored in the cell wall, which is then transformed into chitosan by chitin deacetylase (Zhao et al., 2010).

The quality and quantity of chitosan extracted from fungal mycelia depend on the microorganism, type of fermentation, culture medium composition, pH, temperature and extraction time (Nwe and Stevens, 2004; Zhang et al., 2014). Microorganisms can produce chitosan from synthetic media with defined composition, causing increase in production costs, as in the case of glucose. The use of unconventional substrates like agro-industrial waste is an alternative for obtaining biomolecules from low-cost carbon sources (Vendruscolo et al., 2009b).

Substrates such as soybeans, beans and corn (Suntornsuk et al., 2002; Jiang et al., 2011, Cardoso et al., 2012) and residues from the distillation of *shochu* (Yokoi et al., 1998) have already been used for fungal chitosan production. In Brazil, the state of Santa Catarina is the largest apple producer, producing about 800 thousand tons per year (Vendruscolo et al., 2012), generating large amounts of solid waste, including apple pomace, which is composed of a mixture of bark, pulp and seed generated from the processing of apples to obtain juice, jellies, candies and flavorings (Vendruscolo et al., 2008; Vendruscolo et al., 2009a).

Apple pomace has high moisture content, insoluble carbohydrates such as cellulose, hemicellulose and lignin, reducing sugars

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like glucose and fructose and other sugars such as sucrose, low protein content, essential amino acids, vitamins and minerals (Vendruscolo et al., 2009b). Due to its high content of sugars, apple pomace can be a potential substrate, which in addition to adding value to this agro-industrial waste reduces the environmental impact by avoiding its disposal into the environment.

Airlift bioreactors are a special class of pneumatic bioreactors and have shown greater potential due to the absence of mechanical moving parts and low energy consumption and low shear rate levels when compared to stirred tank bioreactors (Chisti, 1989). Excessive shear may damage suspended cells leading to the loss of viability and even disruption, owing to the influence on the morphology of cell growth caused by hydrodynamic forces (Kawase and Moo-Young, 1990). The magnitude of the shear rate has had important implications in cultures involving fragile animal and plant cells and filamentous microorganisms.

Therefore, this study aimed to use apple pomace as a substrate to produce fungal chitosan by *Gongronella butleri* CCT 4274 in an airlift bioreactor.

2. Material and methods

2.1. Microorganism

G. butleri CCT 4274 was obtained from "André Tosello" Tropical Culture Collection (Campinas – SP, Brazil). The culture was maintained on potato dextrose agar tubes and Roux bottle (Biolife Italiana, Milan, Italy) sterilized at 121 °C for 15 min, incubated at 30 °C for 7 days and subsequently stored at 4 °C.

2.2. Culture medium

Apple pomace (moisture: 73%, reducing sugars: 11.32%, nitrogen: 0.62%, soluble protein: 4.97% and ash: 3.07%) donated by Yakult S. A. (Lages – SC, Brazil) was kept in plastic bags at −20 °C. The culture medium preparation consisted of thawing apple pomace and drying in a forced aeration oven for 24 h at 60 °C. After drying, the material was ground and 35 g were added to 315 mL of distilled water (∼10% w/v) in 1000 mL Erlenmeyer flasks and kept at 60 °C under agitation of 100 rpm for 3 h. The mixture was filtered, the solid residue was discarded and the apple pomace extract (APE) was stored at −20 °C for cultivation in an airlift bioreactor. All cultivations were carried out with initial reducing sugar concentration of 35 ± 1 g L^{-1} added by nitrogen source $NaNO_3$ or $(NH_4)_2SO_4$ at concentration of 5 g L^{-1}.

2.3. Inoculum preparation

A spore suspension was obtained by washing the Roux bottle cultures with a sterile aqueous solution of 0.1% Tween 80. Fungal mycelia was obtained by germination of the spores suspended in 1000 mL baffled flask containing 360 mL of inoculum culture medium (APE) at 30 °C, and shaken on a rotary shaker at 120 rpm for 60 h. This suspension was used for further inoculation in an airlift bioreactor.

2.4. Airlift bioreactor

With the aim of studying the effects of specific aeration (0.1, 0.3 and 0.6 vvm) and nitrogen source $NaNO_3$ and $(NH_4)_2SO_4$ on chitosan production by *G. bulteri* CCT 4274 using apple pomace extract as a substrate, six cultivations were carried out in an airlift bioreactor (volume of 6.5 L) with external loop circulation. After being sterilized using steam at 100 °C for 60 min, the bioreactor was fed with 5.85 L of APE growth medium previously sterilized in autoclave at 121 °C for 15 min, which was inoculated with 0.65 L of inoculum (10% v/v; ≈0.5 g L^{-1} equivalent dry weight). The cultivation was carried by 27 h °C and the pH was maintained at 4.5 with HCl or NaOH 10% (w/v).

2.5. Analytical determinations

2.5.1. pH

The pH was determined through direct measurement in WTW pH meter model pH330i.

2.5.2. Reducing sugars

The concentration of reducing sugars was determined by the 3,5-dinitrosalicylic acid (3,5- DNS) method, as described by Miller (1959). Aliquots of 1.5 mL were centrifuged in *eppendorf* tubes at 1000 rpm for 8 min and the supernatant was collected for analyses. Absorbance was read in a Spectronic Unicam spectrophotometer model Genesis *10vis* at 540 nm. All determinations were performed in triplicate.

2.5.3. Chitosan extraction

Chitosan extraction was performed according to modified methodology proposed by Synowiecki and Al-Khateeb (1997). The biomass dried at 55 °C for 24 h was treated with 2% NaOH (1:30 w/v) at 90 °C for 2 h under agitation of 150 rpm. The insoluble fraction was collected by filtration through filter paper and washed with distilled water and 95% ethanol. The solid material was treated with 10% acetic acid (1:10 w/v) at 60 °C for 6 h with stirring at 150 rpm. The liquid fraction containing chitosan was separated by centrifugation at 3500 rpm for 5 min. Chitosan was precipitated by adjusting the solution pH to 11 with 40% NaOH, and the precipitate was separated by centrifugation at 3500 rpm for 5 min and washed with distilled water and 95% ethanol. Chitosan was submitted to drying in the oven at 90 °C to constant weight.

2.5.4. Degree of deacetylation (DD)

The degree of deacetylation was determined by methodology proposed by Muzzarelli (1996). Chitosan samples dried in the oven at 90 °C were solubilized in acetic acid and submitted to titration with 0.1 M NaOH, yielding a curve with two inflection points. The difference in NaOH volume used between the two inflection points corresponds to the acid consumed for the salification of the amino groups of chitosan, and the degree of acetylation was calculated by

$$DA = \frac{(1 - 161Q)}{(1 + 42Q)} \quad (1)$$

where $Q = N\Delta v/m$, Δv is the NaOH volume used between the inflection points (L), N is the concentration of NaOH solution (mol L^{-1}), m is the dry weight of polymer sample (g) and DA is the degree of acetylation of sample (%), DD is the degree of deacetylation of sample (%) and DA + DD = 100%.

2.6. Kinetic parameters

The cell concentration was determined by filtration of 10 mL of APE growth medium in quantitative Whatman filter paper no. 1 and submitted to drying in microwave oven at power of 180 W for 15 min (Vendruscolo et al., 2010). The maximum specific growth rate was obtained by

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad (2)$$

where μ is the specific growth rate (h^{-1}), X is cell concentration (g L^{-1}) and t is culture time (h). The conversion factor of substrate

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