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Conversion of citric pectin into D-galacturonic acid with high substrate loading using a fermented solid with pectinolytic activity

Daniele Stock Leh[a], Alessandra Biz[b], D.H.F. de Paula[b], Peter Richard[c], Alan Guilherme Gonçalves[d], Miguel Daniel Noseda[d], David Alexander Mitchell[b], Nadia Krieger[a,*]

[a] Departamento de Química, Universidade Federal do Paraná, Cx. P. 19081 Centro Politécnico, Curitiba 81531-980, Paraná, Brazil
[b] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Cx. P. 19046 Centro Politécnico, Curitiba 81531-980, Paraná, Brazil
[c] VTT Technical Research Centre of Finland, Tietotie 2, Espoo 02044, Finland
[d] Departamento de Farmácia, Universidade Federal do Paraná, Av. Lothario Meissner, 3400, Jardim Botânico, Curitiba, Paraná, Brazil

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ABSTRACT

Citrus pulp is a waste product of orange juice processing. Pectin can be extracted from this pulp and used in the food industry. However, citrus pulp is produced in such large amounts that the pectin it contains is far in excess of the current world pectin market. An alternative strategy would be to hydrolyze this excess pectin to liberate D-galacturonic acid (D-galA), which can then be converted into several platform chemicals. We report, for the first time, the hydrolysis of pectin by the direct addition of "pectinolytic fermented solids". These solids were produced by solid-state fermentation of a 30:70 mixture, by dry mass, of ground sugarcane bagasse and orange peels, using a strain of *Aspergillus oryzae* isolated from rotting passion fruit peels. With the addition of this lyophilized fermented solid to a 10% w v^{-1} pectin solution, we obtained 247 mmol L^{-1} of D-galA in the hydrolysate, this being the highest D-galA concentration yet reported. Since the direct addition of fermented solid to the reaction mixture avoids the need for extraction and recovery steps, our process has potential to provide low cost of pectinases for use in citrus-waste biorefineries.

1. Introduction

Citrus pulp, the processing waste of orange juice industries, accounts for almost 50% of the fresh weight of the orange. It is produced in large amounts: Brazil and the United States of America, which are responsible for 80% of the world market, were forecast to produce 1.49 million metric tons of orange juice concentrate in the 2014/2015 harvest (65° Brix) (USDA, 2015), which would result in approximately 2.23 million metric tons of dry citrus pulp. Currently, a portion of the citrus pulp is used as a supplement for cattle feed. However, the moisture content can be as high as 75% w w^{-1}, even after pressing, leading to high drying costs.

Recently, it has been proposed to use citrus waste as a feedstock for biorefineries, with the extraction of limonene and pectin and the conversion of other carbohydrates, such as hemicellulose, cellulose and soluble sugars, to ethanol and biogas (Lohrasbi et al., 2010; López et al., 2010). However, pectin is not a suitable end product for a biorefinery, since the amount of pectin contained in the citrus pulp produced worldwide by the orange juice industry is about an order of magnitude greater than the world pectin market (Kuivanen et al., 2014). An alternative strategy would be to hydrolyze the pectin in citrus pulp to D-galacturonic acid (D-galA), which constitutes about 70% of the mass of citrus pectin (Yapo, 2011), with the subsequent conversion of D-galA into platform chemicals, such as *meso*-galactaric acid (mucic acid) and L-galactonic acid (Richard and Hilditch, 2009). Enzymatic hydrolysis is the preferred strategy for hydrolyzing pectin: the homogalacturonan backbone of the pectin is resistant to acid hydrolysis, and acid treatments that are sufficiently strong to hydrolyze this backbone also degrade a significant proportion of the sugars that are released (Garna et al., 2004). However, the use of enzymatic hydrolysis also has challenges, especially due to the high cost of commercial pectinases and the long hydrolysis times.

Commercial pectinases are produced by submerged fermentation, with it being necessary to recover and concentrate the pectinases from the fermentation broth. One possible way to reduce production costs is to use solid-state fermentation (SSF) (Biz et al., 2016; Heerd et al., 2012). The current work aims to show proof of concept of a strategy that has the potential to reduce costs further, but which has not previously been used with pectinases, namely the direct addition of the fermented solid produced by SSF to the reaction medium. This strategy

* Corresponding author.
E-mail address: nkrieger@ufpr.br (N. Krieger).

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has shown promising results with lipases in esterification and transesterification reactions for the production of biodiesel esters (Fernandes et al., 2007; Soares et al., 2013; Zago et al., 2014). We show that the direct addition of fermented solids does give reasonable yields of D-galA from pectin, even at the high substrate loadings that would be used in a biorefinery.

2. Materials and methods

2.1. Microorganism and substrates for solid-state fermentation

The strain used was *Aspergillus oryzae* CPQBA 394-12 DRM 01, originally isolated from decomposing passion fruit peels by Biz et al. (2016) and identified by CPQBA-UNICAMP (Campinas, Brazil). Stock cultures were grown for 3 days on potato dextrose agar slants at 30 °C and then stored at 4 °C.

Sugarcane bagasse was kindly donated by Destilarias Melhoramentos S/A (Jussara, Brazil). Orange peels, resulting from the extraction of fresh orange juice, were obtained at a local restaurant (Curitiba, Brazil). Both substrates were dried, ground in a knife mill and sieved to obtain particles between 1.18 and 1.70 mm. Orange peel particles were washed three times with water at room temperature to remove soluble carbohydrates and then dried again at 60 °C.

2.2. Chemicals and enzymes

Trifluoroacetic acid (TFA) and the standards Glucose (purity > 99%), Glucuronic acid (> 99%), Xylose (> 99%), Rhamnose (> 99%), Mannose (> 99%), Arabinose (> 99%), D-galA (> 97%) and citric pectin (> 74%) were purchased from Sigma-Aldrich (St. Louis, USA). Ethanol (99.5%) and acetic acid (99.7%) were obtained from Biotec (Cotia, Brazil), while sodium acetate, sulfuric acid and 3,5-dinitrosalicilic acid (DNS) were obtained from VETEC (Duque de Caxias, Brazil). Pectinex Ultra SP-L (9500 PGU mL^{-1}) was kindly provided by Novozymes (Bagsvaerd, Denmark).

2.3. Determination of D-Galacturonic acid in commercial citric pectin

The D-galA content of the pectin purchased from Sigma was quantified by a combined chemical and enzymatic hydrolysis (Garna et al., 2006). For the initial acid hydrolysis, 100 mg of citric pectin was added to 10 mL of 0.20 mol L^{-1} TFA and incubated for 72 h at 80 °C. The pH of the hydrolysate was adjusted to 4.5 with 0.1 mol L^{-1} NaOH. It was then diluted to 25 mL with water and added to 25 mL of 1000-fold diluted Pectinex Ultra SP-L. The mixture was incubated on an orbital shaker at 150 rpm, 30 °C for 24 h, then boiled for 15 min and centrifuged at 2200*g* for 5 min. Analysis of the supernatant by HPLC showed that the Sigma citric pectin contained (w w^{-1}): 80.1% D-galA, 5.0% Glc, 12.3% Man + Xyl + Gal and 2.5% Rha + Ara. The D-galA value was used in the calculation of the percentage liberation of D-galA in the hydrolysis experiments.

2.4. Preparation of fermented solids with pectinolytic activity and of crude extract

Mineral salts solution contained (w v^{-1}): K_2HPO_4 0.3%, $(NH_4)_2SO_4$ 1.3%, $MgSO_4{\cdot}7H_2O$ 0.5%, KCl 1%, $FeSO_4{\cdot}7H_2O$ 0.009%. A spore suspension was prepared by growing *A. oryzae* on potato dextrose agar at 30 °C for three days and collecting the spores with mineral salts solution. The spore concentration in the suspension was determined using a Neubauer chamber.

SSF medium consisted of a 30:70 mixture (dry mass ratio) of ground sugarcane bagasse and orange peels. Each 250-mL Erlenmeyer flask contained 5 g of this medium and was autoclaved at 121 °C for 15 min. Sufficient spore suspension was added to give 10^7 spores per gram of dry solid and then the initial moisture content was adjusted to 70% (wet basis) with mineral salts solution. Flasks were incubated for 12–60 h at 30 °C.

When the fermented solid was lyophilized, this was done for 24 h at −45 °C and 0.1 mbar in a Jouan LP3 Lyophilizer (Allerød, Denmark). The lyophilized fermented solid (LFS) was stored in sealed plastic bags at 4 °C.

Crude extract was obtained by adding 100 mL of acetate buffer (0.2 mol L^{-1}, pH 4.5) per gram of fermented solid (for both fresh and lyophilized fermented solid, see Section 3.2) and incubating the mixture on an orbital shaker at 30 °C, 180 rpm for 30 min. The crude extract was filtered through sterile gauze to remove solids and stored at 4 °C.

Activity values reported for the crude extracts obtained in the fermentations represent the means of measurements made on samples removed from triplicate flasks. Activity values reported for the stability of the LFS also represent the means of measurements made on samples removed from triplicate flasks.

2.5. Determination of pectinolytic activity

To determine the pectinolytic activity in the crude extract, 0.25 mL of the crude extract was added to 0.25 mL of a 0.5% w v^{-1} citric pectin solution (prepared in 0.2 mol L^{-1} acetate buffer pH 4.5) and incubated for 40 min at 37 °C. To determine the pectinolytic activity of the LFS, 20 mg of LFS was added to 1.6 mL of 0.5% w v^{-1} citric pectin and 1.6 mL of acetate buffer (0.2 mol L^{-1}, pH 4.5) and incubated for 40 min at 37 °C, after which the hydrolysate was boiled for 5 min and centrifuged at 2200*g* for 10 min. In both cases, 0.5-µL samples of the hydrolysate were transferred into test tubes containing 0.5 mL of DNS reagent and the liberated reducing sugars were quantified using the DNS method (Bernfeld, 1955). Blank assays were performed for both the crude extract and the fermented solid in the same manner as described above, except that the pectin solution was replaced with acetate buffer (0.2 mol L^{-1} pH 4.5) and DNS was added immediately. This gave zero time absorbances, which were subtracted from the readings obtained in the assays. Reducing sugar concentrations were estimated as D-galA equivalents, using a calibration curve constructed with D-galA. One unit of enzymatic activity corresponds to the liberation of 1 µmol of D-galA equivalents per min. In all cases, the results are expressed on the basis of the original grams of dry solid in the sample (written as U g^{-1}).

2.6. Hydrolysis of pectin using lyophilized fermented solid

The hydrolysis experiments were done in 125-mL Erlenmeyer flasks containing 50 mL of pectin solution (2%, 10%, 15% and 20% w v^{-1}) in acetate buffer (0.2 mol L^{-1}, pH 4.5) and LFS (2, 6, 9 and 12 g, respectively). Flasks were incubated on an orbital shaker at 35 °C, 180 rpm, for 72 h. The experiment done to test the effect of adding sodium azide involved pectin solutions of 10% w v^{-1}, each with 6 g of LFS, incubated with or without sodium azide (0.02% w v^{-1}) on an orbital shaker at 35 °C, 180 rpm, for 48 h. In all cases, the liberated D-galA was quantified by HPLC and expressed both as a concentration in the hydrolysate (mmol L^{-1}) and as a percentage of the total D-galA present in the citric pectin (which, as explained in Section 2.3, contained 80.1% D-galA by weight). The values reported for this hydrolysis experiment represent the means of measurements from triplicate flasks.

2.7. HPLC analysis

Samples of 3 mL were removed during the hydrolysis reaction described in Section 2.6, boiled for 10 min, centrifuged at 2200*g* for 5 min, and then filtered through a 0.45-µm PVDF membrane. The concentrations of D-galA and neutral sugars were determined using an Agilent 1260 Infinity Bio-Inert Quaternary LC System (Santa Clara, USA) fitted with a Cation-H pre-column linked to an Aminex HPX-87H