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# Production of pectinases by solid-state fermentation of a mixture of citrus waste and sugarcane bagasse in a pilot-scale packed-bed bioreactor

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ABSTRACT

Pectinases can be used in citrus waste biorefineries to hydrolyze the pectin in citrus pulp to produce D-galacturonic acid, a potential platform chemical. Solid-state fermentation has the potential to produce low-cost pectinases for such biorefineries, but it is difficult to control the process at large scales. In the current work, *Aspergillus oryzae* was cultivated in a pilot-scale packed-bed bioreactor, on 15 kg of a substrate containing 51.6% citrus pulp and 48.4% sugarcane bagasse (w/w, dry basis). The sugarcane bagasse gave a high bed porosity and ensured a stable bed structure, avoiding problems of bed shrinkage and the formation of compact agglomerates within the bed. As a result, bed temperatures were controlled to within 1 °C of the inlet air temperature and pectinase yields of 33–41 U $g^{-1}$ were obtained across the bed. When the fermented solids were dried and added directly to a pectin solution, they gave a profile for the release of D-galacturonic acid similar to that obtained with a commercial pectinase. These results show the potential for using solid-state fermentation to produce pectinases in a citrus waste biorefinery, with subsequent direct addition of the fermented solids to produce D-galacturonic acid from the pectin contained in the citrus pulp.



## 1. Introduction

Citrus pulp is produced in considerable amounts in Brazil and the USA: in the 2014/2015 harvest, these two countries produced about 1.34 million metric tons of dry citrus pulp (calculated from Ref. [1]). Currently, citrus pulp is either dumped, or given away or sold with a very small margin to be used as a complement for cattle feed [2]. However, citrus waste biorefineries could be established to produce pectin [3], limonene [3–5], ethanol [3–5] and D-galacturonic acid as a platform chemical [6,7].

A key step in citrus wastes biorefineries is the hydrolysis of pectin, liberating D-galacturonic acid. It is necessary to use the enzymatic route for this hydrolysis, since chemical hydrolysis leads to significant degradation of the liberated sugars [8]. However, in order to maximize the economic performance of the biorefinery, it will be essential to minimize the costs of enzymatic pectin hydrolysis. One strategy for doing this is to use solid-state fermentation (SSF) to produce the pectinases. This brings two advantages over producing pectinases by submerged fermentation. First, it is cheaper to produce enzymes by SSF than by submerged fermentation [9]. Second, it is possible to dry the solids at the end of the fermentation, producing so-called "fermented solids", which are then added directly to the reaction mixture of the hydrolysis process, thereby avoiding the steps of recovery and concentration of the enzymes [10].

With respect to pectinase production by SSF, although there have been numerous studies at laboratory scale [11], few attempts have been made to scale up this process. This is not surprising: since general strategies for scale-up of SSF processes are not yet available, processes must be scaled up on a case-by-case basis. In fact, studies of pectinase production involving more than 10 kg of dry substrate have only been carried out by Huerta et al. [12], He and Chen [13] and Pitol et al. [14]. The last of these investigations was undertaken by our group and represents the largest scale

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**Fig. 1.** Physical appearance of the dried substrates used in the fermentations. (a) Sun-dried sugar-cane bagasse; (b) oven-dried citrus pulp.

for production of pectinases by SSF that has been used to date. In that work, *Aspergillus niger* was grown in a pilot-scale packed-bed bioreactor, using 30 kg (dry matter) of a substrate consisting of 90% wheat bran and 10% sugarcane bagasse. However, these fermentations suffered from problems with the formation of agglomerates of substrate particles and with shrinkage of the bed. These problems led to overheating of parts of the bed and a consequent lack of uniformity of pectinase levels within the bed at the end of the fermentation.

With respect to the direct addition of fermented solids with enzyme activity in subsequent processes, this has only been done with lipases: several groups have used "lipolytic fermented solids" to produce biodiesel through the esterification of fatty acids or the transesterification of triacylglycerols, using short chain alcohols [10,15–21]. However, this strategy has not previously been used for the hydrolysis of pectin by pectinases.

In light of these considerations, the current work had two objectives. The first aim was to avoid the problems of overheating of the bed that were experienced in the pilot-scale bioreactor of Pitol et al. [14] by producing pectinases in SSF using a substrate with better mechanical properties, namely a mixture of 51.6% citrus pulp and 48.4% sugarcane bagasse. The organism used was *Aspergillus oryzae*, which produces high pectinase yields on this substrate mixture [22]. The second aim was to demonstrate that pectin can be hydrolyzed by adding dried fermented solids directly to the reaction medium.

## 2. Materials and methods

### 2.1. Raw materials

Sugarcane bagasse and citrus pulp were kindly donated by Usina de Álcool Melhoramentos (Jussara, Brazil) and Corol Cooperativa Agroindustrial (Rolândia, Brazil), respectively. The sugarcane bagasse was sun-dried in the open air for several days while the citrus pulp was dried in an oven with forced ventilation at 70 °C for 24 h. Fig. 1 shows their appearance after drying. Typical compositions of these substrates are given in Table 1 [23,24]. They were used without further preparation.

**Table 1**
Typical compositions of citrus pulp and sugarcane bagasse.

| Component | Composition% (w/w dry mass) | |
|---|---|---|
| | Citrus pulp [23] | Sugarcane bagasse [24] |
| Ether extract | 3.9 | 0.1 |
| Glucose | 14.6 | n.d. |
| Fructose | 15.5 | n.d. |
| Sucrose | 10.9 | n.d. |
| Pectin | 14.4 | n.d. |
| Protein | 7.9 | 2.3 |
| Cellulose | 16.2 | 30.3 |
| hemicellulose | 13.8 | 20.7 |
| ashes | 1.7 | 1.2 |
| lignin | 1.0 | 7.3 |

n.d. = not determined.

**Table 2**
Dimensions of the laboratory-scale columns and the pilot-scale packed bed bioreactor.

| | Laboratory-scale column | Pilot-scale bioreactor |
|---|---|---|
| Bed capacity, dry substrate | 8 g | 15 kg |
| Area of bed cross-section | 9.9 $cm^2$ | 0.42 $m^2$ |
| Bed height | 12 cm | 40 cm |
| Air flow | 100 $cm^3\ min^{-1}$ | 150 $m^3\ h^{-1}$ |
| Superficial velocity | 0.17 $cm\ s^{-1}$ | 0.1 $m\ s^{-1}$ |
| Aeration rate per dry mass | 0.75 $m^3\ h^{-1}\ kg^{-1}$ | 10 $m^3\ h^{-1}\ kg^{-1}$ |

### 2.2. Fungal strain

The strain used was *A. oryzae* CPQBA 394–12 DRM 01, originally isolated from decomposing passion fruit peels and identified by CPQBA–UNICAMP (Campinas, Brazil). It was maintained at 4 °C on potato dextrose agar slants. Before use, it was inoculated onto potato dextrose agar plates and incubated at 30 °C for 4 days for sporulation. Spores were harvested with sterile distilled water, with the spore concentration being determined using a Neubauer chamber.

### 2.3. Solid-state fermentation in the column bioreactor

Throughout this work, moisture contents were determined in an infrared balance (Gehaka IV 2000, São Paulo, Brazil) and are reported on a wet basis. The composition of the solid medium, namely 48.4% of sugarcane bagasse and 51.6% (w/w, dry matter) citrus pulp by mass, was chosen on the basis of an optimization experiment undertaken using a $2^3$ Rotatable Central Composite Design (see the supplementary material for details). This solid medium was autoclaved (121 °C, 15 min), in various beakers, with each beaker containing enough material to load one column, namely 9.16 g of substrate (corresponding to 8 g dry matter). $(NH_4)_2SO_4$ solution was autoclaved (121 °C, 15 min) and added to each beaker to obtain a $(NH_4)_2SO_4$ concentration of 3.48% (w/w, based on total dry substrate). The solid medium in each beaker was inoculated with a spore suspension to obtain a concentration of $4 \times 10^7$ spores per gram of dry substrate and mixed thoroughly. The moisture content after inoculation was 78.4% (w/w). Initially, SSF was performed in glass columns (internal diameter of 4 cm and height of 21 cm). Each column was packed with the inoculated solid from one beaker and placed in a water bath set at 30 °C. A continuous stream of air was injected into the humidifying chamber under each column (Fig. 2) at a rate of 100 $cm^3\ min^{-1}$ (Table 2). A column was removed at each sampling time, with the fermentation being terminated at 48 h in the first column fermentation and