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Operation of a fixed-bed bioreactor in batch and fed-batch modes for production of inulinase by solid-state fermentation

Viviane Astolfi[a], Jaderson Joris[a], Ricardo Verlindo[a], J. Vladimir Oliveira[a], Francisco Maugeri[b], Marcio A. Mazutti[c], Debora de Oliveira[a], Helen Treichel[a,d,*]

[a] Department of Food Engineering, URI – Campus de Erechim, P.O. Box 743, CEP 99700-000, Erechim, RS, Brazil
[b] Department of Food Engineering, FEA/UNICAMP, P.O. Box 6121, CEP 13083-862, Campinas, SP, Brazil
[c] Department of Chemical Engineering–Federal University of Santa Maria, Av. Roraima, 1000, Santa Maria, CEP 97105-900, Brazil
[d] Laboratory of Biochemical Engineering, College of Chemistry and Food Engineering, Federal University of Rio Grande (FURG), P.O. Box 474, Rio Grande-RS 96201-900, Brazil

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ABSTRACT

This work is focused on the inulinase production by solid-state fermentation (SSF) in a fixed-bed reactor (34 cm diameter and 50 cm height) with working capacity of 2-kg of dry substrate operated in batch and fed-batch modes. It was investigated different strategies for feeding the inlet air in the bioreactor (saturated and unsaturated air) as alternative to remove the metabolic heat generated during the microbial growth by evaporative cooling. The kinetic evaluation of the process carried out in batch mode using unsaturated air showed that the evaporative cooling decreasing the mean temperature of the solid-bed, although the enzyme production was lower than that obtained using saturated air. Results showed that maximum enzyme activity ($586 \pm 63\,U\,gds^{-1}$) was obtained in the fed-batch mode using saturated air after 24 h of fermentation. The enzymatic extract obtained by fed-batch mode was characterized and presented optimum temperature and pH in the range of 52–57 °C and 4.8–5.2, respectively. For a temperature range from 40 to 70 °C the enzyme presented decimal reduction time, *D*-value, ranging from 5748 to 47 h, respectively. For a pH range from 3.5 to 5.5 the enzyme showed good stability, presenting *D*-values higher than 2622 h. In terms of Michaelis–Mentem parameters were demonstrated that the crude inulinase activity presented higher affinity for substrate sucrose compared to inulin.

1. Introduction

Inulinases are enzymes potentially useful on production of high fructose syrups (HFS) by enzymatic hydrolysis of inulin, conducting to a yield of 95%, and for production of fructoligosaccharides, compounds with functional and nutritional properties for use in low-calorie diets, stimulation of *Bifidus* and as a source of dietary fiber in food preparations [1,2]. Although the inulinases could be obtained from vegetable and animal sources, microorganisms are the best sources for commercial production of inulinases mainly due to their easy cultivation and high yields of product. So far, it has been found that the microorganisms which can produce high level of inulinases include *Aspergillus* sp. [3,4], *Penicillium* sp. [5], *Staphylococcus* sp. [6], *Streptomyces* sp. [4], *Kluyveromyces* sp. [7–11], *Cryptococcus* sp. [12] and *Pichia* sp. [13–16].

Over the last two decades, a considerable growing in use of solid-state fermentation (SSF) towards enzymes production of interest to food industries has been observed. Studies for inulinase production available in the literature makes use of both solid-state fermentation (SSF) [10,11,14,15,17,18] and submerged fermentation (SMF) [3,12,13,18]. Taking into account the relevance of proposing strategies for the scale up of fermentative processes for enzymes production, just a few works were found in the literature concerning to the inulinase production in pilot-scale bioreactor.

Mazutti et al. [19] evaluated the kinetics of inulinase production by solid-state fermentation in a packed-bed bioreactor with working capacity of 2-kg (dry basis) operated in batch mode. From the results it was verified that the outlet temperature reach values about 50 °C that is deleterious to microbial growth. In addition, the microorganism consumed about 90% of the fermentable sugar in the first 9–10 h of fermentation, limiting the growth.

It is known that the increase of temperature is a drawback of packed-bed bioreactors for solid-state fermentation (SSF). Conventionally, temperature control in SSF is primarily accomplished by

* Corresponding author. Fax: +55 54 35209090.
E-mail address: helen@uricer.edu.br (H. Treichel).

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adjusting the aeration rate. If the temperature is too low, then decreasing the aeration rate enables the temperature to rise due to the respiration of microorganisms. On the other hand, if the temperature of the substrate is high, increasing the aeration rate promotes cooling of the substrate, which is not favorable for the growth of the organism. To compensate for this, air that is partially saturated is used for aeration. This method, called evaporative cooling, of the substrate or the biomass, is effective if uniform aeration exists [20,21].

Another alternative to control the temperature is the use of intermittently mixed bioreactor [22]. This strategy could be employed in association with the operation of the bioreactor in the fed-batch mode. In this sense, the solid substrate could be added at determined time intervals, with consequent mixing of the bed and decreasing the bed temperature, enabling the control of substrate addition. The operation of packed-bed bioreactor in the fed-batch mode could improve the microbial growth, since the substrate is not fully available in the start of the fermentation.

Based on these aspects, the objective of this study was to evaluate the production of inulinase by SSF in a fixed-bed bioreactor using strategies of batch and fed-batch fermentations by the yeast *Kluyveromyces marxianus* NRRL Y-7571. First, preliminary studies were carried out for the production of inulinase by batch and fed-batch modes in small scale. From the results obtained in these tests, 7 experiments were performed in laboratory-scale fixed-bed bioreactor in batch and fed-batch modes to evaluate the inulinase production using different strategies for feeding the inlet air in the bioreactor (saturated and unsaturated air) as alternative to remove the metabolic heat generated during the microbial growth by evaporative cooling. The partial characterization of the crude enzymatic extract obtained by fed-batch mode was also carried out.

2. Materials and methods

2.1. Agroindustrial residues

Sugarcane bagasse was obtained from COTREL Ltd. (Erechim, RS, Brazil), corn steep liquor (CSL) from Corn Products Brazil (Mogi Guaçu, SP, Brazil), soybean meal from Olfar SA (Erechim, RS, Brazil), and sugarcane molasses from Ester Refinery (Campinas, SP, Brazil). The sugarcane molasses was pre-treated before its use following the methodology described by Sguarezi et al. [23]: the pH of cane molasses (200 g L^{-1} solution) was adjusted with sulfuric acid 1–5.0 N. The solution was set to rest at 24 °C for 24 h. The medium was then centrifuged at 5000 × *g* for 15 min, and the final pH was adjusted to 4.0 with NaOH 1.0 M.

2.2. Microorganism and fermentation medium

The strain of *K. marxianus* NRRL Y-7571 used for inulinase production was maintained on YM agar medium (g L^{-1}): yeast extract 3.0, malt extract 3.0, peptone 5.0, glucose 10.0, agar 20.0, and subcultured every 3 weeks.

Cell production for pre-inoculum was carried out in 50 mL test tubes with 10 mL of liquid YM medium. This was inoculated with a loopfull of the stock culture and incubated at 30 °C for 24 h. Each test tube (10 mL) with YM medium was transferred to a 500 mL Erlenmeyer flask with 100 mL of pre-inoculum medium and incubated in orbital shaker at 30 °C and 150 rpm for 24 h. The medium was composed by (g L^{-1}): sucrose 20.0, yeast extract 5.0, K_2HPO_4 5.0, NH_4Cl 1.5, KCl 1.15, and $MgSO_4 \cdot 7H_2O$ 0.65. The cell mass in the pre-inoculum was determined by a direct weight measurement before its use in the fermentation [24].

2.3. Fed-batch process

2.3.1. Fed-batch in small scale

Fermentation runs were carried out in conical flasks using different feeding strategies. The final mass of dry bagasse was set to 5 g and the medium was supplemented with (wt%): CSL 20.0 and soybean bran 5.0. Each flask was covered with hydrophobic fabric and autoclaved at 121 °C for 20 min. After cooling, the flasks were inoculated with 6 mL of cell suspension (10^8 cells g^{-1}) and incubated for 36 h in a chamber with controlled temperature and moisture content. The temperature of incubation and substrate moisture were 36 °C and 65% (w/w), respectively. The feeding strategies were 25, 33.3, 50 and 100 wt% of the final mass of the flask (5 g) and the additions were carried out at 6, 12, 18 and 24 h of fermentation, depending on the employed strategy.

2.3.2. Fed-batch in packed-bed bioreactor

The medium composition was optimized in a previous work by Mazutti et al. [10] as following: 2 kg of sugarcane bagasse supplemented with pre-treated cane molasses 15% (w/w), corn steep liquor (CSL) 30% (w/w), and soybean bran 20% (w/w). The mean diameter of particle size of bagasse was 0.688 mm, where 90% of the particles were retained in sieves with mean aperture diameter from 1.98 to 0.5 mm.

The packed-bed bioreactor consists of a cylindrical stainless bed (34 cm diameter and 50 cm height), and two different strategies of aeration were used: air humidified, which supplies air at relative humidity of 95–100%, and air dried, that consists on a resistance connected in the entrance of the bioreactor. For both strategies, the air enters in the bottom of the bioreactor and passes through the bed, until reaches the exit at the top of it.

The bioreactor was filled with 2 kg of dry sugarcane bagasse, supplemented as described above. The moisture content was set to 65% (w/w) and autoclaved at 121 °C for 20 min. The fermentation runs were started with the inoculation of an optimized volume corresponding to a cell mass of 14 g. All experiments were carried out for 24 h [19].

The inlet and outlet air temperatures were continuously monitored by a temperature probe PT100 (NOVUS, Brazil). In addition, the temperature of the moist substrate was monitored at 10, 20 and 30 cm inside the bioreactor. The respiratory metabolism of the microorganism was evaluated by determining the CO_2 production. The outlet air from the bioreactor was analyzed by a CARBOCAP GMT220 sensor of silicon (VAISALA, Inc., Finland) and its operation is based on the NDIR Single-Beam dual wavelength principle. The temperature probes and CO_2 sensor were connected to an acquisition board (FIELDLOGGER NOVUS, Brazil) with an interval of 1 min among each acquisition. The sugar concentration, moisture content and inulinase activity were evaluated at four different bed heights. Three samples of 100 g were collected at 0–10 cm, 10–20 cm, 20–30 cm and 30–40 cm, which correspond to zone 1, 2, 3, and 4 inside the bioreactor, respectively.

2.4. Inulinase activity assay

After fermentation, the enzyme was extracted from the sugarcane bagasse by adding sodium acetate buffer 0.1 mol L^{-1} pH 4.8 in a solid/liquid ratio of 1:10, following incubation at 50 °C and 150 rpm for 30 min. Inulinase activity was assayed in the supernatant after vacuum filtration using a Whatman qualitative filter paper, grade 1. An aliquot of 0.5 mL of the appropriately diluted enzyme source was incubated with 4.5 mL of 2% (w/v) sucrose solution in sodium acetate buffer (0.1 mol L^{-1} pH 4.8) at 50 °C [24]. Reducing sugars released were measured by the 3,5-dinitrosalicylic acid method [25]. A separate blank was set up for each sample to correct the non-enzymatic release of sugars. One unit of inulinase activity was