Biochemical Engineering Journal 97 (2015) 32–39

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Three-phasic fermentation systems for enzyme production with sugarcane bagasse in stirred tank bioreactors: Effects of operational variables and cultivation method

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ARTICLE INFO

Article history:
Received 16 December 2014
Accepted 3 February 2015
Available online 4 February 2015

Keywords:
Enzyme production
Cellulase
Filamentous fungi
Submerged culture
Xylanase
Sugarcane bagasse

ABSTRACT

The high cost of enzymes is one of the main bottlenecks affecting the industrial production of cellulosic ethanol, which therefore requires the development of improved bioprocesses for the manufacture of cellulases. The present work concerns the selection of operating parameters for enzyme production in three-phasic bioreactors, using sugarcane bagasse as substrate. The parameters considered included cultivation method, substrate particle size and pretreatment, agitation speed, and pH. For both shake flask and stirred tank bioreactor (STB), a new sequential cultivation method employing steam explosion pretreated sugarcane bagasse significantly improved enzyme production, compared to conventional submerged fermentation. Larger substrate particle size provided a better support for fungal growth in shake flasks, while smaller particles resulted in greater homogeneity in stirred tank bioreactors. Maximum endoglucanase and xylanase production in the STB were 1599 ± 66 and $4212 \pm 133\ IU\ L^{-1}$, respectively, under sequential cultivation using pretreated bagasse particles smaller than 0.5 mm, agitation speed of 700 rpm, and pH 5.0. The findings provide useful information concerning the influence of operational variables on (hemi) cellulases production in STB three-phasic cultivations, which should contribute to the development of bioprocesses using lignocellulosic materials in large-scale bioreactors.

1. Introduction

Improvements in second generation (2 G) ethanol production require consideration of environmental and economic issues, including the dependence on petroleum-based fuels and the addition of value to agricultural and industrial residues. A critical factor in many biorefining approaches for 2 G ethanol production is the high cost of the enzymes required for enzymatic hydrolysis of lignocellulosic biomass, which can have a significant impact on the price of 2 G ethanol [1,2]. The development of improved bioprocesses for enzyme production is therefore needed in order to overcome this economic limitation.

The composition of lignocellulosic material favors its use as a renewable resource for the production of biofuels and enzymes. Vegetal biomass is mainly composed of three types of polymer: cellulose (35–50%), hemicellulose (15–35%), and lignin (10–20%). The relative proportions of these substances and the interaction among them vary according to plant species, which affects the recalcitrance of the biomass and its suitability for different purposes [3–5]. When lignocellulosic materials are used as substrates for enzyme production, a pretreatment step can increase cellulose availability. Each type of pretreatment has its advantages and disadvantages. The use of steam explosion helps to remove the hemicellulose fraction from the biomass, rendering the cellulose more available to biological and enzymatic attack. This procedure is widely used for sugarcane bagasse, because no chemicals are required (except water), energy inputs are low, and the technique is compatible with existing biorefinery systems [4,6,7].

Many microorganisms are able to degrade lignocellulosic materials by producing enzymatic complexes containing cellulases (cellobiohydrolases, endoglucanases, and β-glucosidases) and

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http://dx.doi.org/10.1016/j.bej.2015.02.004
1369-703X/

Table 1
Chemical composition of untreated and pretreated sugarcane bagasse.

	Untreated sugarcane bagasse[1]	Steam explosion pretreated sugarcane bagasse[2]
		
Glucan (%)	46.6	71.2
Pentosan (%)	26.5	0.5
Lignin (%)	21.7	27.8

[1] Rodriguez-Zuñiga et al. [23].
[2] Cunha et al. [24].

xylanases. Other accessory enzymes are also produced that can increase the accessibility of the cellulose and enhance the hydrolysis of lignocellulosic materials. These include lytic polysaccharide monooxygenases, pectinases, laccases, manganese peroxidase, and lignin peroxidase [3,8,9]. The most widely studied microorganisms are the filamentous fungi *Trichoderma* sp. and *Aspergillus* sp., which are able to secrete enzymes at very high levels [10,11].

Cellulases and hemicellulases can be produced by two conventional fermentation methods: submerged fermentation (SmF) and solid-state fermentation (SSF). The submerged fermentation method has well-developed monitoring and control systems and is relatively simple to scale up, and has therefore been widely used industrially for cellulase production. On the other hand, solid-state fermentation has the advantage of being able to provide environments similar to the natural habitats of fungi, and close interaction between the microorganism and the inducer substrate can result in higher enzyme yields [12,13]. A new sequential fermentation (SF) method (SF) recently developed by Cunha et al. [14] combines the advantages of the solid-state and submerged cultivation techniques in a single process, and can potentially be used for cellulase production in either shake flasks or pneumatic bioreactors [15].

The bioreactors used for submerged enzyme production can be pneumatically agitated, as in bubble column bioreactors, or mechanically agitated, such as in stirred tank bioreactors (STB) [16,17]. The STB is currently the type of bioreactor most widely used in cellulase production bioprocesses. Although commercial substrates have often been used for cellulase production, there is increasing use of lignocellulosic materials for the production of cellulases and xylanases in bioreactors [15,18,19].

Fungal growth under different environmental conditions can result in differences in physiology and enzyme expression, as well as in the expression of enzymes with different characteristics in terms of molecular weight, kinetic parameters, and performance [13,20]. The influence of the lignocellulosic substrate particle size in solid-state fermentations for enzyme production has been reported recently [21,22]. However, no research has been found concerning the effect of the solid substrate particle size when employed as inducer substrate in submerged fermentation in bioreactors.

Although there has been extensive research into enzyme production, further investigation is required of the influence of operational variables such as particle size, cultivation method, and agitation conditions in stirred tank bioreactors using sugarcane bagasse as substrate. These parameters are of key importance in the design of bioprocesses for large-scale enzyme production. In order to contribute to the development of bioprocess engineering for (hemi) cellulolytic enzymes production, the present work therefore investigates the influence of cultivation method (sequential solid-state and submerged), sugarcane bagasse pretreatment and particle size, agitation speed, and pH control on (hemi) cellulases production by *Aspergillus niger* in a stirred tank bioreactor.

2. Materials and methods

2.1. Substrate

The inducer substrate used was sugarcane bagasse. The *in natura* untreated sugarcane bagasse (USB) was provided by Edra Ecossistemas (Ipeúna, Brazil) and the steam explosion pretreated sugarcane bagasse (PSB) was provided by CTC (Piracicaba, Brazil). Both samples originated from the same region in the State of Sao Paulo (Brazil), and the steam explosion pretreatment was conducted at 17×10^5 Pa and 205 °C for 20 min. Compositional analyses of the untreated and pretreated sugarcane bagasse samples were performed previously by Rodriguez-Zuniga et al. [23] and Cunha et al. [24], respectively. The chemical compositions of the samples are provided in Table 1. The sugarcane bagasse samples were milled and sieved, and particle sizes (described in terms of the characteristic diameter, d_p) were selected in the ranges $d_p \leq 0.5$, $0.5 \leq d_p \leq 1.0$, $1.0 \leq d_p \leq 2.0$, and $2.0 \leq d_p \leq 2.4$ mm.

2.2. Microorganism

The wild type *Aspergillus niger* A12 strain, isolated from black pepper, was obtained from the Embrapa Food Technology collection (Rio de Janeiro, Brazil) [25]. The strain was kept at −18 °C, and was activated by incubation in slants of potato dextrose agar medium for 7 days at 32 °C. Suspensions of spores were prepared by the addition of 10 mL of Tween-80 (0.3%, v/v), and the spore concentrations were determined using a Neubauer chamber.

2.3. Nutrient medium

The nutrient medium used for the pre-culture and enzyme production was adapted from Mandels and Sternberg [26] and contained (w/v): 0.14% $(NH_4)_2SO_4$, 0.20% KH_2PO_4, 0.03% $CaCl_2$, 0.02% $MgSO_4 \times 7H_2O$, 0.50% peptone, 0.20% yeast extract, 0.03% urea, 0.10% Tween 80, and 0.10% of salt solution (5 mg L^{-1} $FeSO_4 \times 7H_2O$, 1.6 mg L^{-1} $MnSO_4 \times H_2O$, 1.4 mg L^{-1} $ZnSO_4 \times 7H_2O$, and 2.0 mg L^{-1} $CoCl_2$). The nutrient medium was supplemented with sugarcane bagasse according to the experimental conditions described in the next sections.

2.4. Experimental design strategies

Two experimental design approaches were carried out in order to investigate the effect of different operational and environmental conditions on enzyme production: statistical factorial design, followed by analysis of variance (ANOVA), and the one-variable-at-a-time (OVAT) method.