available at www.sciencedirect.com

ScienceDirect

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

Induction of cellulases and hemicellulases from *Neurospora crassa* under solid-state cultivation for bioconversion of sorghum bagasse into ethanol

*Ioannis Dogaris, George Vakontios, Emmanuel Kalogeris, Diomi Mamma, Dimitris Kekos**

Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 9 Iroon Polytechniou Street, Zografou Campus, 157 80 Athens, Zografou, Attica, Greece

ARTICLE INFO

Article history:
Received 13 June 2008
Received in revised form
24 July 2008
Accepted 30 July 2008

Keywords:
Neurospora crassa
Solid-state cultivation
Cellulase
Hemicellulase
Wheat straw
Sorghum bagasse
Bioethanol

ABSTRACT

The cellulolytic and hemicellulolytic system from the mesophilic fungus *Neurospora crassa* was produced under solid-state cultivation (SSC) on wheat straw and wheat bran mixtures. Following optimization of nitrogen source, pH and initial moisture of the growth medium, yields as high as 492.8, 1.08, 26.7, 297.8 and 0.132 (in U g^{-1} of carbon source) were obtained for endoglucanase, exoglucanase, β-glucosidase, xylanase and β-xylosidase, respectively. The potential of the multienzyme system was demonstrated for hydrolysis of sorghum bagasse (SB) into fermentable carbohydrates. *N. crassa* cells were found able to assimilate the majority of the released sugars and generated limited levels of other metabolic products during simultaneous saccharification and fermentation of this valuable substrate into ethanol.

1. Introduction

In view of recent developments in crude oil market prices, the use of alternative, non-petroleum based sources of energy is expected to rise sharply in the coming years (Kumar et al., 2008). Lignocellulosic biomass (energy crops) and wastes (forest, agricultural, and municipal) could offer a huge renewable resource for second generation biofuels production (Hahn-Hägerdal et al., 2006; Tengerdy and Szakacs, 2003). Efficient and inexpensive production of cellulolytic and hemicellulolytic systems for enzymatic breakdown of such materials could accelerate the forthcoming changes and provide a solution that is consistent with the increasing public environmental concern (Öhgren et al., 2007; Zhang et al., 2006). Meanwhile, commercial cellulases and hemicellulases are used in a different, though wide, range of applications, including detergents and textile industry, pulp and paper industry, animal feeding, extraction of fruit and vegetable juices, and starch processing (Bhat, 2000; Beg et al., 2001; Polizeli et al., 2005).

Numerous fungi have been identified to degrade cellulose and hemicellulose, but only a few exhibit both depolymerase and fermentative capacity (Lezinou et al., 1995). *Neurospora crassa* is able to synthesise and secrete high levels of all three

* *Corresponding author*. Tel.: +30 2107723205; fax: +30 2107723161.
E-mail address: kekos@chemeng.ntua.gr (D. Kekos).
0926-6690/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.indcrop.2008.07.008

enzyme types involved in cellulose degradation (Yazdi et al., 1990), as well as endoxylanase and β-xylosidase activities (Mishra et al., 1984; Deshpande et al., 1986). In addition, it is a well-known ethanol producing microorganism that has been used for fermentation of agricultural residues (Rao et al., 1985). The application of biological systems for direct microbial conversion of lignocellulosic materials into ethanol is highly advantageous due to significant reductions in both capital and operation cost (Cardona and Sánchez, 2007; Wyman, 2007).

Production of enzymes by solid-state cultivation (SSC) represents an attractive alternative over conventional submerged cultivation (Viniegra-González et al., 2003). The advantages of SSC, which have been claimed by many workers, include higher productivity per reactor volume, lower cost and space requirements, simpler equipment and easier downstream processing (Pérez-Guerra et al., 2003). The use of SSC for induction of cellulolytic and hemicellulolytic enzymes provides a means to exploit various agro-industrial by-products, such as wheat bran, wheat straw, corn cobs sugar beet pulp, apple pomace, and cassava waste (Pandey et al., 1999).

Utilization of sorghum bagasse (SB), the solid residue obtained after extraction of sugars from sweet sorghum stalks, is important for the economy of the global use of the crop (Negro et al., 1999). Scenarios for SB exploitation are based on enzymatic hydrolysis of polysaccharides and co-fermentation of glucose and xylose to ethanol (Gnansounou et al., 2005). However, previous attempts to take advantage of this valuable bioethanol industry derivative were associated with relatively low ethanol yields (Ballesteros et al., 2004).

The aim of the present work was to optimize production of cellulolytic and hemicellulolytic enzymes from *N. crassa* under SSC of agro-industrial by-products. Furthermore, a two-phase approach (SSC followed by anaerobic fermentation) for conversion of SB to ethanol was attempted. Important variables of the process were explored, including the ability of the enzymatic system to break down polymers into simple fermentable sugars, and the capacity of fungal cells to assimilate them without producing undesirable metabolic products.

2. Materials and methods

2.1. Microorganism

The microorganism used in the present study was *N. crassa* DSM 1129 and supplied by DSMZ (Deutsche Sammlung von Microorganismen und Zellkulture, Germany). Stock cultures were maintained on potato dextrose agar slants at 4 °C.

2.2. Reagents

Carboxymethyl cellulose, birchwood xylan and *p*-nitrophenyl glycosides were obtained from Sigma Chemical (St Louis, MO, USA). All other chemicals were analytical grade.

2.3. Solid substrates

Wheat straw (WS), wheat bran (WB), corn cobs (CC) and fresh sweet sorghum stalks were chopped to less than 3 mm diameter particles. SB was prepared following double extraction of sugars from sweet sorghum in 15% (w/v) aqueous suspension at 50 °C for 45 min, filtration, extensive washing and drying at 70 °C overnight.

2.4. Media and growth conditions

SSC was carried out in 100-ml Erlenmeyer flasks containing 2.5 g of dry carbon source moistened with Toyama's mineral medium (in g l^{-1}: $(NH_4)_2SO_4$, 10; KH_2PO_4, 3; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.5) (Toyama and Ogawa, 1978). Following heat sterilization (121 °C) for 20 min, each flask was inoculated with 1 ml spore suspension (approximately 5.8×10^7 conidia) and incubated at 30 °C under static conditions. Experiments were carried out in duplicate.

A step-by-step optimization procedure regarding the effect of important parameters (carbon and nitrogen source, initial growth pH and moisture) on enzymes production was employed. Ammonium sulphate, ammonium phosphate, potassium nitrate, urea, yeast extract, peptone and corn steep liquor were the nitrogen sources examined. Their elemental concentration was 0.04 g nitrogen per g of carbon source. The initial culture pH ranged between 4.0 and 7.0. The moisture level (60.0, 70.0, 80.0 and 87.5% (w/w)) was adjusted with the addition of the appropriate amount of Toyama's mineral medium.

2.5. Enzyme extraction

After suitable periods of time, enzymes were extracted from the fermented growth medium with 10-fold (v/w) citrate–phosphate buffer pH 5.0 (50 mM) by shaking (250 rpm) at 28 °C for 60 min. The suspended materials and fungal biomass were separated by centrifugation (12,000 × *g* at 4 °C for 15 min) and the clarified supernatant was used for enzyme activity measurements.

2.6. Enzyme assays

Endoglucanase (EG), exoglucanase (EXG) and xylanase (XYL) activities were assayed on carboxymethyl cellulose, Avicel and birchwood xylan, respectively, as described (Kalogeris et al., 1999). Measurement of released reducing sugars was accomplished by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The activities of β-glucosidase (β-GLU) and β-xylosidase (β-XYL) were determined spectrophotometrically using the respective *p*-nitrophenyl glycosides as substrates (Kalogeris et al., 1999). All assays were carried out at 50 °C and pH 5.0. Blanks with inactivated enzyme (after boiling for 15 min) were used as a reference. One unit (U) of enzyme activity was defined as the amount of enzyme liberating 1 μmol of product per min.

2.7. Biomass estimation

Monitoring microbial growth during SSC was based on glucosamine content of fungal cell wall, which was determined by the colorimetric method of Ride and Drysdale (1972). Conversion into biomass was achieved using a coefficient factor estimated with *N. crassa* cells grown on 2% glucose, and results were expressed as mg of biomass per g of dry substrate.