Bioresource Technology 101 (2010) 6432–6437

Contents lists available at ScienceDirect

Bioresource Technology

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

Cellulose and cellodextrin utilization by the cellulolytic bacterium *Cytophaga hutchisonii*

Yongtao Zhu, Huihui Li, Hong Zhou, Guanjun Chen, Weifeng Liu *

The State Key Laboratory of Microbial Technology, School of Life Science, Shandong University, Jinan 250100, Shandong, China

ARTICLE INFO

Article history:
Received 6 December 2009
Received in revised form 28 February 2010
Accepted 10 March 2010
Available online 1 April 2010

Keywords:
Cytophaga hutchinsonii
Cellulose
Soluble sugar
Cellodextrins
Membrane

ABSTRACT

Cytophaga hutchinsonii is an abundant aerobic cellulolytic soil bacterium utilizing very few substrates as sole carbon and energy sources. In this study, growth of *C. hutchinsonii* on different substrates including crystalline cellulose, regenerated amorphous cellulose (RAC) as well as soluble sugars including cellodextrins was analyzed. Soluble sugars including glucose and cellodextrins were produced extracellularly when *C. hutchinsonii* was cultured on cellulose. Preferential use of cellulooligosaccharides as the carbon source by *C. hutchinsonii* was largely dependent on its inoculation status. Compared with glucose-grown cells, inoculation of cellobiose-grown cells led to a rapid assimilation of cellobiose or cellodextrins with longer-chain cellodextrins being hydrolyzed extracellularly to smaller oligomers during the culture. Further analysis of the distribution of cellulase activity revealed that, while the carboxymethylcellulase activity significantly induced by crystalline cellulose was highest in the outer membrane, the cellobiase activity was highest in the cytoplasmic membrane. These results suggest that membrane-bound cellulases may play an important role in cellulose solubilization by *C. hutchinsonii* and that metabolism of cello-oligosaccharides is a tightly coupled step in this process.

1. Introduction

Cellulose, a principal component of plant cell walls, consists of linear polymers of β-1,4-linked glucose molecules that are organized into higher order fibrillar structures. As the most abundant biomass in nature, its decomposition not only plays a key role in the carbon cycle of nature, but also provides a great potential for a number of applications, most notably biofuel production (Lynd et al., 2002, 2005). Microbial degradation of cellulose involves a complex interplay between different cellulolytic enzymes. Among others, it has been widely accepted that three types of cellulases including endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) act synergistically to convert cellulose to glucose (Lynd et al., 2002). A broad range of microorganisms secreting these activities either in separate enzymes or in multi-protein complexes have been described. Extensive evidence obtained from aerobic cellulolytic microorganisms supports a hydrolysis mode mediated by synergistic action by endoglucanases and cellobiohydrolases with cellobiose as the main product of cellulose solubilization (Divne et al., 1994; Zhang and Lynd, 2004). Evidence has also been presented indicating that cellodextrins other than cellobiose are the predominant products from cellulose degradation by some anaerobic microorganisms (Russell, 1985; Zhang and Lynd, 2005). Efficient assimilation of these compounds followed by phosphorolytic cleavage has been thought to be of great bioenergetic benefit and therefore critical for the survival of the cellulolytic microbes (Lynd et al., 2002; Russell, 1985; Zhang and Lynd, 2005). Moreover, cellodextrin cross feeding between cellulolytic and non-cellulolytic microbes has been proposed to help explain the high numbers of non-cellulolytic bacteria in the rumen of animals (Russell, 1985). Cellodextrin utilization in aerobic cellulolytic microorganisms has not been extensively studied.

Cytophaga hutchinsonii is an abundant aerobic cellulolytic soil bacterium which was originally isolated by Walker and Warren from soil (Walker and Warren, 1938). Although *C. hutchinsonii* has been found to be an efficient aerobic degrader of crystalline cellulose such as filter paper, cellulose wadding, cellophane and cotton wool (Stanier, 1942), most of the cellulase activity seems to be cell-associated (Chang and Thayer, 1977; Stanier, 1942; Xie et al., 2007), and physical contact between cells and cellulose substrate appears to be necessary for the efficient hydrolysis of cellulose. Besides cellulose, cellobiose and glucose are the other known substrates utilizable by *C. hutchinsonii* (Larkin, 1989). Analysis of the genomic sequences of *C. hutchinsonii* has revealed no obvious homologs of known cellobiohydrolases and processive endoglucanases which are thought to be critical in the degradation of crystalline cellulose (Wilson, 2008; Xie et al., 2007). Therefore, a different mode of action from either the noncomplexed cellulase systems produced by aerobic microorganisms or the complexed

* Corresponding author. Fax: +86 531 88565610.
E-mail address: weifliu@sdu.edu.cn (W. Liu).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.03.041

cellulase systems possessed by anaerobic microorganisms has been proposed for *C. hutchinsonii* (Wilson, 2008; Xie et al., 2007).

In this study, growth of *C. hutchinsonii* on different substrates including crystalline cellulose, regenerated amorphous cellulose (RAC) and soluble sugars including cellodextrins was analyzed. The impact of inoculation status on cellulooligosaccharide utilization was evaluated. The induction of relative cellulase activities by different substrates and their cellular localization were also determined.

2. Methods

2.1. Microbial strain and culture conditions

C. hutchinsonii ATCC 33406 was grown in PY2 medium containing (per liter) 2.0 g peptone, 0.5 g yeast extract. The pH of this medium was adjusted to 7.3 with NaOH. The carbohydrate sources for growth included 0.1% (wt/vol) glucose, 0.1% (wt/vol) cellobiose, 0.1% (wt/vol) cellodextrins, 0.3% (wt/vol) crystalline cellulose, or 0.35% (wt/vol) regenerated amorphous cellulose (RAC). All incubations were done in 100 ml flasks with shaking (180 rpm) at 28 °C.

2.2. Substrate preparation

RAC was prepared as described by Zhang et al. (2006) with some modifications to scale up the preparation. Briefly, 10 g of crystalline cellulose was mixed with 500 ml of 86.2% H_3PO_4 and left on ice for 1 h with occasional stirring. Approximately 1.5 L of ice-cold distilled water was added to the mixture. The precipitated swollen cellulose was washed extensively by ice-cold distilled water followed by adjusting pH to 7.0 with 2 M Na_2CO_3. A portion of the final RAC suspension was dried to determine the concentration.

Cellodextrins were prepared by mixed-acid hydrolysis of crystalline cellulose and chromatographical separation essentially as described by Zhang and Lynd (2003) with some modifications. Briefly, 10 g of crystalline cellulose was suspended in 40 ml of ice-cold HCl (~37%), and 20 ml of ice-cold H_2SO_4 (98%) was added slowly to the slurry. The mixture was stirred at room temperature for 4 h. Approximately 900 ml of acetone (−20 °C) was added to the mixture followed by vigorous stirring. The solution was centrifuged at 10,000*g* at 4 °C for 15 min. The pellet was washed three times with ice-cold acetone and dissolved in 300 ml of distilled water. The water-soluble cellodextrin solution was then applied to a Bio-Rad AG1_X8 (OH-type) anion-exchange column (0.7 by 10 cm) to remove Cl^- and SO_4^{2-}. The resultant solution was concentrated by a rotary evaporator and the residual acetone was removed simultaneously. The concentrated cellodextrin solution (approximate 50 mg/ml) was centrifuged to remove insoluble longer cellodextrins and loaded onto a Bio-Rad AG50 W-X4 column (5 by 27 cm) arranged in series with a Bio-Gel P4 column (5 by 95 cm). The columns were eluted using distilled water with a flow rate of 1.5 ml/min while column temperature was maintained between 60 and 70 °C. Total sugars in the eluting fractions were determined using the modified phenol sulfuric acid method (Masuko et al., 2005) (Fig. 1a). The purity of separated cellodextrins was determined by high-pressure liquid chromatography (Fig. 1b).

2.3. Growth with soluble sugars or cellulose

Growth of *C. hutchinsonii* was monitored by measuring the increase in optical density (600 nm) with a SPECTRAMAX 190 microplate spectrophotometer (Molecular Devices Corporation, USA) when glucose, cellobiose or cellodextrins were used as the carbon source. Residual sugars in cultures were determined by HPLC. When crystalline cellulose or RAC were used as carbohydrate sources, cellular proteins were determined to reflect growth as described elsewhere (Jun et al., 2007; Thomas and Russell, 2004). Briefly, samples (30 ml) including both bacteria and residual cellulose were pelleted by centrifugation at 10,000*g* for 10 min. The pellet was washed once with 0.9% NaCl (wt/vol), dried at 65 °C to a constant weight before being boiled for 20 min in 0.2 M NaOH. Residual cellulose particles were removed by centrifugation and protein in the supernatant was determined by the method of Bradford, 1976. The weight of residual cellulose equals the total weight of the pellet minus the dry weight of cells. Total soluble sugars in cultures were determined by the phenol sulfuric acid method (Masuko et al., 2005).

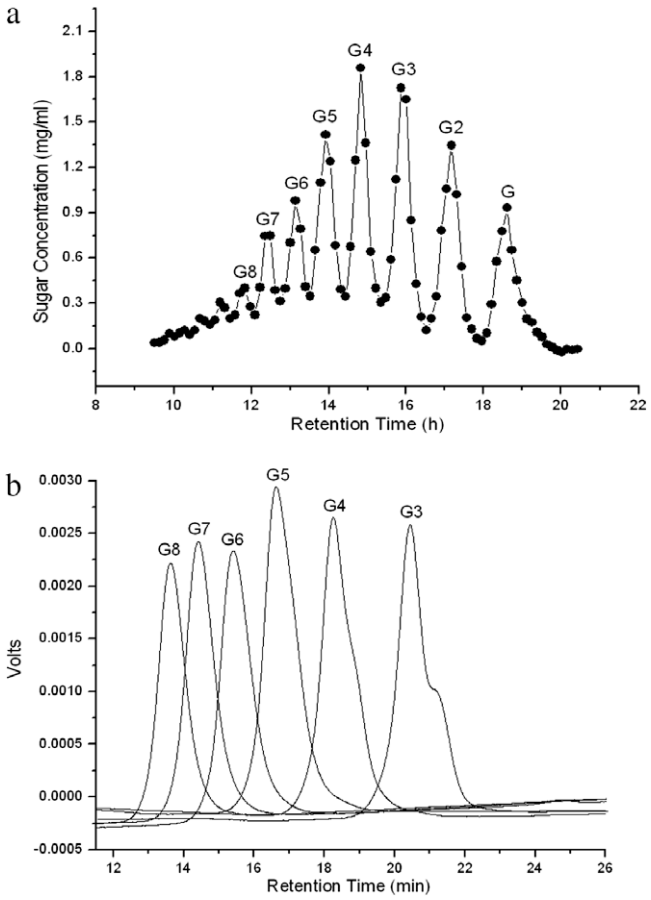

Fig. 1. Cellodextrin preparation. (a) Cellodextrins separated by preparative columns. (b) HPLC analysis of purified individual cellodextrins with DP from 3 to 8. Purities of G3, G4 and G5 were all above 95%.

2.4. Cellulose hydrolysis by resting cells

Essentially, resting cells were prepared as described by Kilbane (1989). Cells from exponentially growing cultures (1 L) were harvested (6000*g*, 10 min), washed twice with 0.9% NaCl, resuspended in 10 ml of 20 mM potassium phosphate buffer (pH 7.0) and stored at 4 °C. Resting cell suspension (1 ml) was mixed with 10 ml of 20 mM potassium phosphate buffer containing 0.5% (wt/vol) crystalline cellulose. The mixture was kept at 28 °C and samples (1.0 ml) were withdrawn at different time intervals and supernatants were analyzed by HPLC after cells were removed by centrifugation.