Screening of carbon sources for beta-glucosidase production by *Aspergillus saccharolyticus*

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ABSTRACT

The fungus *Aspergillus saccharolyticus* was found to produce a culture broth rich in beta-glucosidase activity, an enzyme which plays an essential role for efficient and complete hydrolysis of lignocellulosic biomass. Direct application of fungal fermentation broths produced on-site in a biorefinery may be an integral part of a biorefinery for lowering the cost associated with the use of commercial enzymes for saccharification of biomass. Utilization of low value slip streams from the biorefinery as substrates for such an on-site enzyme production would be ideal to reduce costs. In order to understand which carbon sources that support growth and trigger *A. saccharolyticus* to produce beta-glucosidases, carbon sources, ranging from monomer sugars to complex lignocellulosic biomasses, including pretreated and hydrolyzed corn stover fractions, were investigated as substrates and inducers of enzyme production. A convenient micro titer plate experimental setup was developed that facilitated a fast screening for beta-glucosidase activity on the different carbon sources. The greatest beta-glucosidase activity was found when *A. saccharolyticus* was cultivated on media containing xylose, xylan, wheat bran, and pretreated corn stover. In a refinery, beta-glucosidase production by *A. saccharolyticus* could with success be based on the biomass hemicelluloses and their degradation products which cannot be converted by conventional yeast.

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1. Introduction

Filamentous fungi grow in many different habitats in nature, where they have adapted to various conditions. The ability of a specific fungus to survive in a given habitat relies on its ability to use the available carbon sources. Plant biomass, consisting mainly of polysaccharides with varying amounts of lignin and proteins, is an important carbon source for a wide range of fungi, being either saprotrophs, plant pathogens or plant symbionts. These complex plant polymers cannot be taken up directly by the fungi, but are degraded by a complex mixture of enzymes released by the fungi (Perez et al., 2002). Such fungal enzymes are key components in biomass degradation and are also exploited in biorefinery processes for sustainable production of biofuels and biochemicals (Perez et al., 2002).

Commercial lignocellulytic enzymes most often of fungal origin are constantly being optimized to perform better individually as well as together with the aim of obtaining higher hydrolysis yields at lower enzyme loading. However, enzymes are still considered an expensive process step in the conversion of lignocellulosic biomass to biofuels and chemicals (Lau et al., 2012). On-site enzyme production using lignocellulolytic fungi can help close the value chain of a biorefinery and advance sustainable biofuels and biochemicals (Rana et al., 2014). Using cheap substrate solutions as the fermentation medium would be an advantage, such as the use of substrates and slip streams produced within the biorefinery. The choice of growth medium should support growth and fungal biomass accumulation, while the induction medium should trigger enzyme expression (Lau et al., 2012). Increased knowledge on how to induce and increase the production of enzymes is of major importance for an economically feasible production for industrial applications. Lignocellulolytic enzymes have been reported to be induced or repressed by a variety of carbon sources, ranging from sugar monomers to lignocellulosic biomasses (Stricker et al., 2008; Noguchi et al., 2011). Even though production of the enzymes is

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generally induced by their substrates, the actual inducers are likely mono- or disaccharides as fungi cannot take up polysaccharides. With the exception of glucose mediated catabolite repression, the compounds that often induce the production of a specific enzyme is the product or a metabolic derivative of the enzyme reaction (de Vries, 2003). Therefore, to be able to determine the actual inducer of the production of lignocellulolytic enzymes, it is important to screen single lignocellulosic cell wall compounds such as monomer sugars and oligomers along with the complex biomasses.

Among lignocellulolytic enzymes, beta-glucosidases (EC 3.2.1.21) play a key role for the complete hydrolysis of cellulose (Sørensen et al., 2013). They perform the final hydrolysis step to release the glucose monomers and during that event relieve the inhibition of cellobiohydrolases and endoglucanase by hydrolyzing cellobiose and cellodextrins thus facilitating even greater hydrolysis performance of the complete enzyme cocktail (Perez et al., 2002). *Aspergillus saccharolyticus* strain AP has recently been found to produce new and highly efficient beta-glucosidases that proved to be well suited for the hydrolysis of plant biomass (Sørensen et al., 2011a,b; Rana et al., 2014). The fungus was originally isolated from oak wood, indicating that the fungus has the enzymatic capability of degrading and accessing carbon sugars from complex plant biomass. In the present study, carbon sources, ranging from monomer sugars to complex lignocellulosic biomasses that supported growth of the fungus and were stimulating for production of beta-glucosidases were investigated as a step towards applying *A. saccharolyticus* for on-site enzyme production in a biorefinery process. A convenient micro titer plate experimental setup that facilitated a fast screening for beta-glucosidase induction on the different carbon sources was developed. Finally, pretreated and hydrolyzed corn stover fractions were explored for their potential as substrates for on-site enzyme production.

2. Materials and methods

2.1. Fungal strain and spore suspension preparation

A. saccharolyticus strain AP (CBS 127449) (Sørensen et al., 2011a) was cultured on potato dextrose agar (Sigma Aldrich) and spores were harvested after 7 days of growth, using sterile water. The spore suspension was filtered through two layers of Miracloth (Andwin Scientific, USA). A hemocytometer (iN CYTO, USA) was used for cell counting.

2.2. Media and carbon sources

All chemicals were purchased from Sigma Aldrich, unless specified otherwise.

Basic minimal Czapek medium (without carbon source) contained 3 g/l $NaNO_3$, 1 g/l K_2HPO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l KCl, 0.1 g/l $FeSO_4 \cdot 7H_2O$ (Samson et al., 2004). 2% w/v of different carbon sources was added, and 1.5% agar was used for growth on solid medium. Initial pH was adjusted to 4.5.

Complete medium (without carbon source) contained 4 g/l peptone, 40 g/l corn steep liquor, 2 g/l yeast extract, 2 g/l casamino acids, 12 g/l $NaNO_3$, 1 g/l KCl, 1 g/l $MgSO_4 \cdot 7H_2O$, 3 g/l KH_2PO_4, 0.1 g/l Na_4 EDTA, 4.5 mg/l $ZnSO_4$ $7H_2O$, 22 mg/l H_3BO_3, 10 mg/l $MnCl_2 \cdot 4H_2O$, 10 mg/l $FeSO_4 \cdot 7H_2O$, 3.4 mg $CoCl_2 \cdot 6H_2O$, 3.2 mg/l $CuSO_4 \cdot 5H_2O$, 0.17 mg/l $Na_2MoO_4 \cdot 2H_2O$. Different carbon sources were added to this medium, see later descriptions.

All pure carbon sources (mono-, di-, polysaccharides) were purchased from Sigma Aldrich. A description and literature based composition of the lignocellulosic biomasses used is given in Table 1.

2.3. Growth and enzyme activity on solid medium

Growth on different carbon sources was evaluated by placing a 25 μl droplet of spore solution (10^6 spores/ml) in the center of an agar plate with Czapek medium and carbon source. The plates were prepared in triple determination and incubated at 28 °C, and morphological assessment was done on day 5.

To determine enzyme activity, 25 μl spore solutions were spread out covering agar plates with Czapek medium and carbon source. The plates were prepared in triple determination and incubated at 28 °C for 5 days. Three plugs of a 1 cm diameter were cut from each plate; the plugs were further cut in smaller pieces, placed in Eppendorf tubes with 1 ml sterile water and incubated at 4 °C for 24 h to extract the enzymes, similar to the method described in Pedersen et al. (2009). Samples were centrifuged for 5 min at 10 000 g and supernatants were tested for enzyme activity and protein content.

2.4. Enzyme induction in liquid micro-assay

Two hundred ml potato dextrose broth in a 1 L baffled shake flask was inoculated with spores (final concentration 10^6/ml), and incubated at 28 °C, 140 rpm for 24 h to obtain an actively growing culture. The fungal mycelium was harvested and washed with 1 L sterile water using MiraCloth as sieve. The mycelium was resuspended in 400 ml double concentrated Czapek liquid medium (without carbon source). Aliquots of 750 μl were distributed in the wells of 96 deep well 2 ml plates (Fisherbrand). Seven hundred fifty μl of 4% w/v carbon source in water was added to each well to obtain a final concentration of 2% w/v carbon source. The plates were covered with double layered MiraCloth and incubated at 28 °C, 120 rpm for 4 days. The plates were centrifuged at 4000 rpm

Table 1
Lignocellulosic carbon sources.

Carbon source	Description	Composition			
		Cellulose	Hemicellulose	Lignin	References
Oatmeal		11%	10%	3%	(Fahrenholz, 1997)
Wheat bran	The hard outer layer of wheat cereals	10−15%	25−30%	4−8%	(Maes and Delcour, 2001; Phyllis2)
Corn cob	The central core of maize	30−45%	32−40%	5−15%	(Dominguez et al., 1997; Phyllis2; Saha, 2003)
Corn stover	Leaves and stalks of maize	32−36%	19−22%	11−15%	(Templeton et al., 2009; Weiss et al., 2010)
Wheat straw	The dry stalks of wheat cereal plants	45−55%	25−35%	15−20%	(Phyllis2)
Bagasse	The fibrous matter that remains after sugarcane stalks are crushed to extract their juice	40−45%	24−35%	18−30%	(Miyamoto, 1997; Sun et al., 2004; Cardona et al., 2010)
Empty fruit bunch	A left-over from palm oil mill processing	23%	21%	29%	(Phyllis2)
Orchard grass		30%	14%	5%	(Phyllis2)
Laminaria		1−7%	5−10%	0%	(Ross et al., 2008)