Enzyme and Microbial Technology 43 (2008) 48–55

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Trichoderma atroviride mutants with enhanced production of cellulase and β-glucosidase on pretreated willow

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ARTICLE INFO

Article history:
Received 13 December 2007
Received in revised form 8 February 2008
Accepted 13 February 2008

Keywords:
Trichoderma atroviride
Cellulase production
β-Glucosidase production
Pretreated willow
Fermentation
Enzymatic hydrolysis of cellulose

ABSTRACT

Trichoderma atroviride mutants were developed by using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) treatment and UV-light followed by a semiquantitative plate clearing assay on Walseth-cellulose/agar plates. The parent strain of the mutants was an isolate from the Amazonas basin (TUB F-1505), whose identity was established by ITS 1 and 2 and *tef1* gene sequence analysis. Strain F-1505 proved to be the most promising extracellular cellulase producer among 150 wild-type *Trichoderma* in a screening program performed in shake flask fermentation on pretreated willow. Reducing sugar content, soluble protein, filter paper cellulase activity (FPA), β-glucosidase activity and endoglucanase activity of the fermentation broths of the mutant strains were measured in both shake flask and lab-scale fermenters and compared with *Trichoderma reesei* Rut C30. Also, hydrolytic capacities of fermentation supernatants of *T. reesei* Rut C30, the parent strain (F-1505) and the best mutants were compared on pretreated willow as carbon source and hydrolysis substrate. The *T. atroviride* mutants produced high levels of extracellular cellulases as well as β-glucosidase, rendering the need for β-glucosidase supplementation in hydrolysis of cellulose or pretreated willow unnecessary. On the contrary, β-glucosidase supplementations were essential in order to obtain good glucose yields for all other cellulase preparations tested.

1. Introduction

During the last four decades a lot of effort has been devoted to develop mutants capable of producing efficient cellulase enzymes in high quantities. *Trichoderma viride* QM 6a was initially selected for cellulase production by Mandels and Weber in the late 60s based on screening of more than 100 wild-type *Trichoderma* isolates [1]. This strain was later identified as a new species and named *Trichoderma reesei* in honor of E.T. Reese. The parent strain QM 6a was further developed by chemical mutagenesis and irradiation at the US Army Natick Laboratories to produce a mutant named QM 9414 that had higher filter paper activity (FPA) than the wild-type strain [2]. Similar methods have been used in other laboratories to produce hypercellulolytic *T. reesei* mutants, such as L27 (Cetus Corporation, USA) [3], VTT-D-80133 (VTT, Finland) [4] and CL-847 (Cayla, France) [5].

The most common method for selection of hypercellulolytic *Trichoderma reesei* mutants was developed by Montenecourt and Eveleigh using Petri plate screening with Walseth-cellulose, colony growth inhibitor and catabolite repressor [6,7]. Mutagenesis was carried out using UV-light and nitrosoguanidine and a range of mutants were isolated [8]. *Trichoderma reesei* Rut C30, probably the best known mutant was selected as the strain of choice as it produced 4–5 times more cellulase than the wild-type QM 6a.

Although *T. reesei* produces the CBH and EG components of cellulase enzyme complex in high quantities, its β-glucosidase activity in the culture filtrate is low. In order to achieve good cellulose hydrolysis *T. reesei* cellulases are in most cases supplemented with β-glucosidase enzymes from *Aspergillus*. β-Glucosidase production of the mutants was not investigated by Mandels et al. [2] when they selected the best isolate for cellulase production. The biosynthesis and the role of β-glucosidase enzymes in the saccharification of cellulose were first studied by Sternberg [9]. In 2002 US Department of Energy (DOE) supported two leading enzyme manufacturers (Novozymes, Genencor) to enhance the productivity of industrial mutants. Both companies invested in further development of *T. reesei* mutants in order to increase β-glucosidase production [10]. Hypercellulolytic strain development to date has focused almost solely on a single species of *Trichoderma* (*T. reesei*) even

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doi:10.1016/j.enzmictec.2008.02.006

though there are at least 45 different *Trichoderma* species currently identified and characterized [11–13].

For the economical production of bioalcohol from plant materials, the improvement of cellulase secreting microorganisms is of vital importance. To date research has focused on cellulase-producing microbes originally selected on pure celluloses that are not substrate targeted to break down natural or pretreated lignocellulose materials. Since pure cellulose is a relatively expensive substrate, for large scale production of cellulase inexpensive natural lignocellulosic materials, pretreated lignocelluloses [14,15] or lactose [16–18] are preferable. More efficient and substrate specific cellulase-producing mutants are needed that are very productive on natural lignocellulosics or on pretreated lignocellulosic materials. The enzyme complexes also have to be very efficient for the hydrolysis of such inexpensive lignocellulose substrates. To our knowledge no research has been carried out using cheap pretreated lignocellulose materials (instead of pure cellulose) in order to select the best candidate *Trichoderma* strain for mutation.

Our research hypothesis was that *T. reesei* originally selected at Natick Laboratories based on its productivity on pure cellulose may not always be competitive on cheap lignocellulosic materials with other, still untested cellulolytic fungi. *Trichoderma reesei* QM 6a (the wild parent of best existing hypercellulolytic mutants) grew scarcely on pretreated lignocellulose and produced only moderate amount of cellulase. The goal of this study was to perform a screening for cellulase production in shake flask among 150 wild-type *Trichoderma* isolates on a lignocellulosic substrate (steam pretreated willow), which is one of the candidate substrates in the Swedish bioalcohol program. The selection criteria included both good filter paper degrading activities and β-glucosidase production. The best wild-type strain was further mutated to enhance cellulase secretion on the same substrate. Lab-scale agitated fermentation experiments and hydrolysis studies were also performed.

2. Materials and methods

2.1. Trichoderma isolates

The majority of wild-type *Trichoderma* strains investigated came from the TUB (Technical University of Budapest, Hungary) collection. These strains had been isolated from soil samples (worldwide), the colonies had been purified and the pure cultures had been freeze-dried. *Trichoderma reesei* QM 6a was purchased from Quartermaster collection (Massachusetts, USA). *Trichoderma reesei* Rut C30 was kindly donated by Prof. D. E. Eveleigh (Rutgers University, New Jersey, USA). Revitalization of freeze-dried cultures was performed on potato-dextrose-agar (PDA) Petri plates at 30 °C. Sporulated cultures were used for inoculation. Altogether approximately 150 *Trichoderma* isolates coming from 30 countries were tested. The identity of many of the isolates has been determined in previous studies [13,19–21].

2.2. Preparation of mutants

Before mutation, total living spore number of a Petri plate culture of *Trichoderma* strains was determined by serial dilutions, propagation on PDA + 0.5% Triton X100 medium and colony counting. Mutants were prepared by both UV-irradiation and chemical mutagenesis. For UV-mutagenesis the spores from a Petri plate culture were scraped and suspended in 100 ml of sterile water containing 0.1% Tween-80. The spore suspension was then transferred to Petri dishes to a depth of ~3 mm. The time and distance of irradiation as key parameters were altered in order to obtain 99% lethality. Cellulase secretion of colonies were visualized by a semiquantitative plate assay [6], namely the treated spore suspensions were spread in 0.1 ml quantities on the surface of Walseth-cellulose agar plates and incubated at 30 °C for 4–7 days. Walseth-cellulose was prepared as described by Tansey [22]. The agar media used for the plate clearing assay contained (in g/l) Walseth cellulose, 5; glycerol, 50; $NaNO_3$, 2; $(NH_4)_2SO_4$, 1; $(NH_4)_2HPO_4$, 1; Tween-80, 1; KH_2PO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; $CaCl_2$, 0.5; Bacto yeast extract, 0.5; Triton X100, 5; agar, 15 and (in mg/l): $CoCl_2{\cdot}6H_2O$, 2; $MnSO_4$, 1.6; $ZnSO_4{\cdot}7H_2O$, 3.45; $FeSO_4{\cdot}7H_2O$, 5.

Two research protocols have been used for *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) mutagenesis. In the first one, 9 ml of spore suspension was treated with 0.1% NTG to 99% lethality, then 0.1 ml quantities were spread on Walseth-cellulose plates. After incubation at 30 °C for 4–7 days, positive colonies were point inoculated into the mid-part of potato-dextrose agar (PDA) Petri dishes and after incubation they were used in the shake flask fermentation experiments (30 °C, 3 days) on pretreated willow. In the second mutagenesis method, 100 ml of spore suspension was treated with 0.1% NTG to 99% lethality, and this treated suspension was directly used for the inoculation of shake flask media. After fermentation (30 °C, 3 days), cultures with the highest cellulase activities were plated in 0.1 ml quantities on PDA medium supplemented with 0.3% Triton X100 and incubated at 30 °C for 6 days. Single colonies representing different and sometimes unique colony morphologies were point inoculated onto PDA dishes and were later retested in shake flasks. Primary colonies were transferred twice to the PDA agar medium and stable mutants were freeze-dried.

2.3. Pretreated willow

Willow, a fast-growing energy crop in Sweden was found to be a suitable carbon source for cellulase production [23,24] and a good raw material for ethanol production from wood [25,26]. Lignocellulosic materials are very resistant to enzymatic attack, therefore, prior to use they have to be pretreated in order to make the cellulose more accessible to enzymes.

Chopped willow chips were impregnated with sulfur dioxide (3% w/w, moisture) for 20 min at room temperature. The impregnated material was steam pretreated at 205 °C for 5 min in a steam-pretreatment unit equipped with a 10-l reactor vessel [27]. The slurry was thoroughly mixed and stored at 4 °C until use. Total dry matter (DM) and water-insoluble solid (WIS) content of the pretreated willow were determined.

Representative samples of the steam pretreated slurry were withdrawn for analysis. Soluble sugars, degradation products and total soluble sugars after acid hydrolysis were determined from the liquid fraction according to the standardized methods of the National Renewable Energy Laboratory (NREL, Golden, CO, USA) [28]. The solid residue was washed with water in order to remove all soluble substances, and the composition of the WIS was determined according to Sluiter et al. [29].

2.4. Shake flask fermentation

Two different media containing pretreated willow (1W and 2W) and two pure cellulose containing media (N and E1) with different types of cellulose were used for the shake flask experiments. The compositions were as follows:

Medium 1W (in g/l): pretreated willow (whole slurry), 15 (total dry matter); KH_2PO_4, 2; $(NH_4)_2SO_4$, 2; proteose peptone (DIFCO), 1; Tween-80, 1; paraffin oil (antifoam), 0.5; $MgSO_4{\cdot}7H_2O$, 0.3; urea, 0.3; $CaCl_2$, 0.3; $CaCO_3$, 1, and (in mg/l): $CoCl_2{\cdot}6H_2O$, 2; $MnSO_4$, 1.6; $ZnSO_4{\cdot}7H_2O$, 3.45; $FeSO_4{\cdot}7H_2O$, 5. The pH before sterilization was 5.8.

Medium 2W (in g/l): pretreated willow (whole slurry), 15 (total dry matter); KH_2PO_4, 1.5; $(NH_4)_2HPO_4$, 2; soybean meal, 1; corn steep liquor (50% dry matter), 1; NaCl, 0.5; Tween-80, 0.5; paraffin oil (antifoam), 0.5; $MgSO_4{\cdot}7H_2O$, 0.3; urea, 0.3; $CaCl_2$, 0.3; $CaCO_3$, 1, and (in mg/l): $CoCl_2{\cdot}6H_2O$, 2; $MnSO_4$, 1.6; $ZnSO_4{\cdot}7H_2O$, 3.45; $FeSO_4{\cdot}7H_2O$, 5. The pH before sterilization was 4.8.

Medium N (in g/l): cellulose powder (Sigmacell Type 20, or Cellulosepulver MN 301, or Solka Floc 40), 10; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; proteose peptone (DIFCO), 1; Tween-80, 1; antifoam, 1; $MgSO_4{\cdot}7H_2O$, 0.3; urea, 0.3; $CaCl_2$, 0.3; $CaCO_3$, 1, and (in mg/l): $CoCl_2{\cdot}6H_2O$, 4; $MnSO_4$, 3.2; $ZnSO_4{\cdot}7H_2O$, 6.9; $FeSO_4{\cdot}7H_2O$, 10. The pH before sterilization was 5.0.

Medium E1 (in g/l): cellulose powder (Sigmacell Type 20, or Cellulosepulver MN 301, or Solka Floc 40), 10; KH_2PO_4, 2; $(NH_4)_2HPO_4$, 1.5; soybean meal, 1; corn steep liquor (50% dry matter), 2; NaCl, 0.5; Tween-80, 1; antifoam, 1; $MgSO_4{\cdot}7H_2O$, 0.3; urea, 0.3; $CaCl_2$, 0.3; $CaCO_3$, 1, and (in mg/l): $CoCl_2{\cdot}6H_2O$, 4; $MnSO_4$, 3.2; $ZnSO_4{\cdot}7H_2O$, 6.9; $FeSO_4{\cdot}7H_2O$, 10. The pH before sterilization was 5.0.

The cellulose powders were purchased from SIGMA (Sigmacell Type 20), from Macherey-Nagel GmbH, Duren, Germany (Cellulosepulver MN 301) and Fiber Sales & Development Corp., Urbana, OH, USA (Solka Floc 40).

Shake flask fermentations were carried out in 750 ml cotton plugged Erlenmeyer flasks containing 150 ml medium. After autoclaving at 121 °C for 20 min the flasks were inoculated by removing the spores from a fully sporulated Petri plate (approx. 10^6 viable spores per ml of shake flask medium). Flask cultivation was performed at 30 °C on a rotary shaker at 220 revolutions per minute. After three days of cultivation, samples were removed and centrifuged at 8000 rpm for 8 min and the clear supernatants were used for enzyme assays.

2.5. Bench-scale agitated fermentation

The experiments were performed in 2-l LABFORS mini fermenters (Infors AG, Switzerland), with a working volume of 1.5 l. Due to foaming only 50% of the whole volume was used as working volume. Medium 2W (composition see above) was used in the experiments, with the difference that instead of paraffin oil, silicone antifoam (SIGMA, Antifoam A Emulsion) was used to control foaming.

Fermenters containing 900 ml of the medium were sterilized in the autoclave (ex situ) at 121 °C for a minimum of 30 min. After cooling, 100 ml of spore suspension was added. All fermentations were performed at 30 °C. The pH was controlled in the range of 4.5–5.8 by addition of 5% NaOH and 5% H_2SO_4. In all experiments the