Enzyme and Microbial Technology 43 (2008) 403–409

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Generation of recombinants strains to cellulases production by protoplast fusion between *Penicillium echinulatum* and *Trichoderma harzianum*

Aldo José Pinheiro Dillon[a,*], Marli Camassola[a], João Antônio Pegas Henriques[a], Maria Helena Pelegrinelli Fungaro[b], Andréia Cristiane Souza Azevedo[b], Tarciso André Ferreira Velho[a], Sergio Echeverrigaray Laguna[a]

[a] *Instituto de Biotecnologia, Universidade de Caxias do Sul, CP 1130, CEP95100-700 Caxias do Sul, RS, Brazil*
[b] *Universidade Estadual de Londrina, CP 6001, CEP86051-990 Londrina, PR, Brazil*

ARTICLE INFO

Article history:
Received 18 February 2008
Received in revised form 23 July 2008
Accepted 29 July 2008

Keywords:
Protoplast
Fusion
Trichoderma
Penicillium echinulatum
Cellulases

ABSTRACT

Nowadays, cellulase importance is growing mainly due to its potential utilization in developing second-generation ethanol technology. Thus, considerable effort has been made to obtain new genotypes with improved capacity to produce efficient and cost-effective enzyme complexes for cellulose hydrolysis. The majority of the genetically manipulated strains that produce higher amounts of cellulases come from of the wild *Trichoderma reesei* QM6A. Such as *T. reesei* cellulolytic mutants, *Penicillium echinulatum* mutants also produce high titles of cellulases (filter paper activities—FPA), and its enzymatic complex effectively hydrolyses cellulose. In the present work, we obtained genetic variability with the purpose of enhancing cellulase production by protoplast fusion between *P. echinulatum* and *T. harzianum*. The resulting fusants, who were all morphologically like the *P. echinulatum* parental, were initially characterized by RAPD, and the results showed that almost all amplified DNA bands showed correlation with *P. echinulatum* DNA bands. Some of new selected strains presented higher FPA and β-glucosidases activities when compared to the parental strains. One particular clone – BP2 – showed values of FPA activities around of 2 UI ml^{-1}, in submerged cultures and all selected strains showed faster and higher secretion of cellulases in solid-state cultures, in comparison to the parental strain of *P. echinulatum*. Altogether, at the same time that our results show increased cellulase secretion by *P. echinulatum* strains, they also show that protoplast fusion techniques associated with an efficient selection process could be a useful tool in genetic improvement to generate better cellulose-producing strains aimed at economically hydrolyzing cellulose with a view to second-generation ethanol.

1. Introduction

Cellulases are enzymatic complex, that comprises exo-β-1,4-glucanases (EC 3.2.1.91), endo-β-1,4-glucanases (EC 3.2.1.4) and β-1,4-glucanases (EC 3.2.1.21), that act synergistically in the hydrolysis of β-1,4-glycosidic bonds present in cellulose polymers. Commercially, these enzymes are mainly used by the textile and detergent industries [1,2]. However, their most significant potential application, which would require high quantities of these enzymes, lies in cellulose saccharification to produce ethanol [3–5]. Significant efforts have been made to understand the mechanisms regulating the expression of genes encoding cellulases [5–9], to obtain genetic variants in improvement programs involving mutagenesis and selection [10–18], and to obtain genetic transformants carrying specific markers of interest [1,19,20].

In addition to mutagenesis and genetic transformation, protoplast fusion can also be used to achieve genetic alterations in cellulases secretion in fungi that consists of the alternative to inducing gene recombination and generate genetic variation [21–26].

The cellulolytic microorganisms are evolutionary "factories" of enzymes and a lot of genes, not yet perfectly identified or known, must operate jointly to achieve an efficient cellulase secretion, especially in view of the composition and structure of lignocellulosic materials. From this point of view, the known cellulase genes and their control system are only a part of all genes, which can be involved with a cellulase secretion. Therefore, all these genes can vary in different species or genera of related microorganisms that secrete cellulases; they could be recombined through protoplast fusion and powerful screening techniques would choose the better recombinant strains of interest.

* Corresponding author. Tel.: +55 54 3218 2100; fax: +55 54 3218 2100.
E-mail address: ajpdillo@ucs.br (A.J.P. Dillon).

0141-0229/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2008.07.009

Protoplast fusion is also a very powerful approach for fast genetic improvement when associated with mutation techniques, as seen with tylosin production by *Streptomyces fradiae* [27].

In addition to *Trichoderma reesei*, *Penicillium echinulatum* is among the microorganisms that show great potential of cellulase secretion for enzymatically converting biomass into ethanol. For instance, mutants of *P. echinulatum* are able to secrete cellulases with filter paper activity (FPA) higher than 2 IU ml^{-1} when grown in media containing 1% cellulose as carbon source [14,15]. They also produce high yields of cellulases in solid-state fermentation using sugar-cane bagasse (SCB) that reach more than 30 FPU g^{-1} of SCB [28]. Moreover, the FPA and β-glucosidases of *P. echinulatum* are of interest for cellulose hydrolysis because they show good thermal stability at 50 °C and 55 °C, respectively [29], and its cellulase complex presents a good proportion of FPA and β-glucosidase for efficiently hydrolyzing cellulose, when compared to the cellulases of *T. reesei* [30].

Nevertheless, it has been difficult to obtain new cellulase mutants of *P. echinulatum* 9A02, although a good methodology has been applied [15]. Due to this, in the present work, we aimed to isolate genetic variants with improved cellulases secretion. For this purpose, we induced genetic recombination by bringing together the genomes of *P. echinulatum* and *Trichoderma harzianum* through protoplast fusion. The *T. harzianum* strain was isolated as an intestinal symbiont of the Coleoptera *Passalus binominatus erosus* and also secretes cellulases, appearing at a faster growth than *P. echinulatum*, but with a smaller production of it (data not shown).

The genetic recombination does not necessary involve cellulase genes of both strains; it is more likely that general genes involved in the growth, if recombined, could produce some new interesting genotypes. Therefore, later after the possible genetic segregation of the heterokaryons, we selected clones that produced faster and clearer halos in the agar plate containing swollen cellulose. Occurrence of genetic recombinantion in fusion products was evaluated by Random Amplification of Polymorphic DNA (RAPD), and the fusants were studied for FPA and β-glucosidases secretion in both submerged fermentation (SF) and in solid-state fermentation (SSF). The new strains obtained showed higher cellulolytic activity in submerged cultures and also faster and higher secretion of cellulases in solid-state cultivations in relation to parental strains.

2. Materials and methods

2.1. Fungal strains

In this work, we used cellulolytic mutant strains 2427, obtained from the wild type (2HH) of *P. echinulatum* after UV radiation and selection in agar plate containing cellulose for observing hydrolysis haloes [14], and the cellulolytic mutants 5A-AS5 (AS5) and 5A36, obtained from the wild type (5A) of *T. harzianum* using the same methodologies used with *P. echinulatum* [31]. Both strains belong to cultures of the collection of the Laboratory of Enzymes and Biomass, Institute of Biotechnology, University of Caxias do Sul, Brazil, which after growth at 28 °C are maintained on slands of cellulose agar (CA) plate at 4 °C and subcultured at 6 months intervals.

2.2. Media

All media used were based on 10X concentrated Mandels mineral solution (MS) [32] containing (g l^{-1}) KH_2PO_4, 20; $(NH_4)_2SO_4$, 3; $CO(NH_2)_2$, 3; $MgSO_4{\cdot}7H_2O$, 3; $CaCl_2$, 3; $FeSO_4{\cdot}7H_2O$, 0.050; $MnSO_4{\cdot}H_2O$, 0.0156; $ZnSO_4{\cdot}7H_2O$, 0.014; $CoCl2{\cdot}6H_2O$, 0.0020. Swollen cellulose gel was produced by placing 5 g of Cellufloc 200 cellulose (Celulose e Amido Ltda, Suzano, São Paulo, Brazil), 60 ml of distilled water and 20 glass beads (ϕ: 3 mm) in a 500-ml Erlenmeyer flask, which was then sealed and autoclaved at 121 °C for 30 min. These flasks were placed in an orbital shaker for 48 h at 180 rpm. Later, 140 ml of distilled water was added and the swollen cellulose was stored at 4 °C until use. Unless otherwise stated, chemicals were purchased from Sigma (São Paulo, SP, Brazil).

To growth and manutention of strains was used the cellulose agar (CA) [15] consisting of 40% (v/v) of swollen cellulose suspension, 10% (v/v) 10 × MS [32], 0.1% (w/v) proteose peptone (Oxoid) and 2% (w/v) agar and to observe halo formation on cellulose-containing dishes we used CA medium supplemented with 0.1% (v/v) of Triton X-100 (TCA) to restrict colony development. When necessary was used the GTCA medium, where glucose (0.5–1.5% final concentration) was added to the TCA medium to produced catabolic repression of cellulases. To obtain mycelia to obtain protoplast and extracted DNA was used the CLM medium constituted of 1% (w/v) of glucose, 10% (v/v) 10 × MS and 0.1% (w/v) proteose peptone.

In order to select the mutants, media were based on CA containing following fungicides: 12.5 μg ml^{-1} of benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate) corresponding to the commercial product benlate from DuPont, Inc. (BCA) and the medium with cycloheximide contained 50 μg ml^{-1} of (3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-glutarimide) (Sigma) (CCA). The medium with nystatin (NCA) contained 30 U ml^{-1} of the commercial product mycostatin of Bristol–Myers Squibb, Inc.

Protoplasts were regenerated in liquid media (PLM) containing 0.6 M of KCl and 10% (v/v) of MS [32]. Heterokaryons were selected on CA medium plus 0.6 M of KCl containing either 12.5 μg ml^{-1} of benomyl and 50 μg ml^{-1} of cycloheximide (BCCA medium), or 12.5 μg ml^{-1} of benomyl and 30 U ml^{-1} of nystatin (BNCA medium).

In the submerged growth for cellulases production, fungi were grown in 500 ml Erlenmeyer flasks containing 100 ml of liquid media containing 20% (v/v) MS × 10 [32], 0.2% (w/v) soy meal, 0.1% (v/v) Tween 80 with 1% (w/v) of glucose (LM) or Cellufloc 200 cellulose (CLM), respectively. The solid-state culture medium consisted of 1 g of wheat bran moistened with 1 ml of 10 × MS [32] and placed in flasks with a 12 cm × 3 cm concave base. The flasks were closed with a gauze-covered cotton-wool plug and autoclaved at 121 °C for 1 h.

2.3. Maintenance of strains and inocula preparation

All strains were grown and maintained on cellulose agar (CA) [15]. Cultures were grown for up to 7 days at 28 °C until conidia formation, and used to inoculate the submerged fermentation (SF) or solid-state fermentation (SSF) or stored at 4 °C.

2.4. Obtaining genetic markers

Spores obtained from 7-day colonies grown in cellulose agar (CA) were used to obtain 10 ml suspension of 2×10^8 conidia ml^{-1}. This conidia suspension disposed in open Petri dish was exposed to ultraviolet radiation (UV 254 nm) using a low mercury pressure Tohawalite—G15T8 germicidal lamp, 15 cm away from the source, with periodic agitation (~40 s) for the amount of time necessary to kill 95% of the cells [15]. Volumes of 0.1 ml of the treated conidia suspension were spread in the CA medium containing benomyl, cycloheximide, or nystatin to isolate clones containing resistance to these substances.

2.5. Protoplast preparation and fusion

To obtain protoplasts, we used 0.2 g of wet mycelia obtained from liquid cultures grown after filtration in the LM medium during 24 h (*T. harzianum*) or 48 h (*P. echinulatum*), being the 0.2 g of mycelia obtained by filtration of liquid culture using Whatman no. 1 Filter Paper and washed one time with distilled water. The mycelia was immediately treated for 3–4 h at 35 °C with 5 ml of Novozym 234® solution (5 mg/5 ml of KCl 0.6 M in a citrate buffer 0.05 M, pH 4.8) or 5 ml of Glucanex® solution (50 mg/5 ml of KCl 0.6 M solution in a citrate buffer 0.05 M, pH 4.8). Protoplast concentration was estimated with use of the hemacytometer and microscope. Suspensions containing equal amounts of protoplast (approximately 1×10^6 protoplasts) from both strains were gently centrifuged at 3000 rpm, and the added of 1 ml of the fusant solution (30% of polypropynoglycol 4000 in a 0.05-M solution of $CaCl_2$ and glycine 0.025 M, pH 8) and maintained for 10 min at 28 °C. The suspensions were then washed with KCl 0.6 M solution and gently centrifuged at 1000 × *g* for 20 min. The resulting pellets were re-suspended in regeneration medium (PLM), and kept under 60 rpm orbital agitation at 28 °C for 24 h. During this period, protoplast germination was followed by observation under the optical microscope. Later, the suspension was spread on selective Petri dishes containing BCCA or BNCA selective media. The dishes were kept at 28 °C for 7 days and the developed colonies were transferred to new selective plate dishes, 1–7 times.

2.6. RAPD Analysis

For DNA extraction, mycelia were obtained from 4-day old cultures (at 28 °C at 180 rpm) grown in 500 ml Erlenmeyer flasks containing 100 ml of CLM medium inoculated with 10^7 conidia. The mycelia were filtered in Whatman no. 1 filter paper and washed three times with distilled water, and either used directly or stored in freezers at −80 °C until needed.

For genomic DNA extraction and RAPD procedures we followed standard protocols as previously described by Ferreira and Grattapaglia [33]. For RAPD, we used commercial primers belonging to the OPERON kit, OPB1-15 and OPX 1-20 kits. The amplification was performed on a Techgene thermal cycler (Techne, Princeton, NJ, USA) by 40 cycles with the following sequence: 92 °C (45 s); 40 °C (1 min and 30 s) and 72 °C (5 min) after 92 °C for 4 min. Amplification products were separated by electrophoresis in 1.5% agarose gel, Tris-borate buffer (0.09 M) and a constant voltage of 100 V, using a Hoefer HE-99 horizontal submarine unit (Hoefer Instruments, San