Available online at www.sciencedirect.com
SCIENCE DIRECT®
Enzyme and Microbial Technology 37 (2005) 82–92
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Response surface modeling of factors influencing the production of chitinolytic and β-1,3-glucanolytic enzymes in *Trichoderma atroviride* strain P1

Bruno Giuliano Garisto Donzelli [a,*], Karl J. Siebert [b], Gary E. Harman [a]

[a] *Department of Horticultural Sciences, Cornell University, Geneva, NY 14456, USA*
[b] *Department of Food Science and Technology, Cornell University, Geneva, NY 14456, USA*

Received 14 October 2004; received in revised form 26 January 2005; accepted 28 January 2005

Abstract

The relationships between five culture factors (glucose, ammonium ion, chitin, scleroglucan, and initial inoculum concentration) and the production of *N*-acetylhexosaminidase, endochitinase, and β-1,3-glucanase enzymatic activities by *Trichoderma atroviride* strain P1 were studied using response surface modeling. Experiments were performed using a 5-factor, 3-level, central composite face-centered design. Enzyme activity levels were modeled using partial least-squares regression (PLS). Enzyme activity levels were strongly affected by both glucose concentration and pH. When the enzyme activity levels were modeled over a range of four factors that excluded wide changes in pH, the most influential factors were chitin and inoculum. The highest production of all three enzyme activities was predicted at glucose concentrations between 0.01 and 0.1%, ammonium ion between 18 and 25 mM, and chitin, scleroglucan, and initial inoculum, at the highest levels tested (0.8, 0.2 and 1×10^7 conidia/50 ml starter culture, respectively).

Keywords: *Trichoderma*; Response Surface; Chitinases; β-1,3-Glucanases; Partial least-squares

1. Introduction

Fungi in the genus *Trichoderma* are prolific hydrolase producers [1]. Enzymes such as *endo*-acting chitinases (E.C. 3.2.1.14), β-*N*-acetylhexosaminidases (E.C. 3.2.1.52), and 1,3-β-glucanases (E.C. 3.2.1.6; E.C. 3.2.1.39; E.C. 3.2.1.58) are strongly fungitoxic [2,3], and for this reason they are considered essential factors for biocontrol by *Trichoderma* [4,5]. These enzymes may also have commercial application as part of antifungal preparations or in the production of nutraceuticals glucosamine and *N*-acetylglucosamine from industrial byproducts (e.g. crustacean exoskeletons) [6,7].

Genes encoding several *Trichoderma* β-1,3-glucanases and chitinases have been isolated. Their expression was found to be under the control of inducers such as chitin or β-glucans, some environmental stresses, and the nitrogen and carbon status of the medium [8,9]. Expression studies at gene, protein, or activity level have almost always been carried out by assessing the effect of one environmental factor at a time. It was of interest to apply response surface modeling to see if a mathematical representation of the effect of relevant culture conditions on production of cell wall degrading enzymes (CWDEs) in *T. atroviride* strain P1 could be developed. If successful, the results could be used to optimize the production of these activities.

2. Materials and methods

2.1. Materials

Chitin from crab shells, 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (MUA), 4-methylumbelliferyl-β-D-*N*,*N*′-

* Corresponding author. Present address: USDA-ARS, U.S. Plant, Soil, and Nutrition Lab, Tower Road, Ithaca, NY 14853, USA.
Tel.: +1 607 255 0271; fax: +1 607 255 2739.
E-mail address: bdd1@cornell.edu (B.G.G. Donzelli).

0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.01.033

Table 1
Experimental conditions and responses from the exploratory design

Run #	Factor levels					Reponses			
	Glu[a] (%)	Amm[b] (mM)	Chi[c] (%)	Scl[d] (%)	Inoc[e] (spores/run)	Nahase[f] (U)	Endo[g] (U)	β13glu[h] (U)	pH
1	0.1	5	0	0	10^7	8.3	3.7	27.0	6.2
2	3	5	0	0	10^5	0.3	0.3	10.8	5.2
3	0.1	100	0	0	10^5	5.2	1.5	20.2	6.0
4	3	100	0	0	10^7	0.1	0.0	9.7	3.5
5	0.1	5	1.5	0	10^5	1.3	1.7	23.8	6.0
6	3	5	1.5	0	10^7	0.1	0.3	10.6	5.5
7	0.1	100	1.5	0	10^7	8.1	16.0	34.3	6.2
8	3	100	1.5	0	10^5	0.0	0.0	8.1	3.5
9	0.1	5	0	0.2	10^5	4.8	0.7	16.1	6.1
10	3	5	0	0.2	10^7	0.4	0.0	7.8	4.9
11	0.1	100	0	0.2	10^7	14.9	6.0	31.1	6.0
12	3	100	0	0.2	10^5	0.1	0.0	10.4	3.8
13	0.1	5	1.5	0.2	10^7	13.6	12.5	34.6	6.1
14	3	5	1.5	0.2	10^5	0.4	0.1	8.0	4.4
15	0.1	100	1.5	0.2	10^5	3.2	3.9	29.5	6.1
16	3	100	1.5	0.2	10^7	0.0	0.0	8.0	3.4
17	0.1	52.5	0.75	0.1	5.1×10^6	22.8	9.6	33.2	6.0
18	3	52.5	0.75	0.1	5.1×10^6	0.0	0.0	7.3	3.2
19	1.55	5	0.75	0.1	5.1×10^6	0.1	0.1	6.5	5.2
20	1.55	100	0.75	0.1	5.1×10^6	0.0	0.0	6.3	5.1
21	1.55	52.5	0	0.1	5.1×10^6	0.1	0.0	6.6	4.1
22	1.55	52.5	1.5	0.1	5.1×10^6	0.0	0.0	7.8	5.4
23	1.55	52.5	0.75	0	5.1×10^6	0.0	0.0	12.6	4.0
24	1.55	52.5	0.75	0.2	5.1×10^6	0.0	0.0	10.9	4.9
25	1.55	52.5	0.75	0.1	10^5	0.0	0.0	8.3	3.9
26	1.55	52.5	0.75	0.1	10^7	0.0	0.0	9.5	4.1
27	1.55	52.5	0.75	0.1	5.1×10^6	0.0	0.0	9.1	4.9
28	1.55	52.5	0.75	0.1	5.1×10^6	0.0	0.0	10.4	3.5
29	1.55	52.5	0.75	0.1	5.1×10^6	0.0	0.0	9.2	3.8

[a] Glucose.
[b] Ammonium ion.
[c] Chitin.
[d] Scleroglucan.
[e] Inoculum.
[f] *N*-Acetylhexosaminidase.
[g] Endochitinase.
[h] β-1,3-Glucanase.

diacetyl-chitobioside, laminarin, aniline blue, and sodium azide were obtained from Sigma (St. Louis, MO). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were from Difco (Detroit, MI). Scleroglucan (a β-1,3/β-1,6-glucan) was obtained from Carbomer (Westborough, MA). Miracloth was purchased from Calbiochem (San Diego, CA). BSA fraction V, KH_2PO_4, $(NH_4)_2HPO_4$, $MgSO_4$, $CaCl_2$, $FeCl_3$, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, $CoCl_2$, $CuSO_4{\cdot}5H_2O$, and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ were purchased from Fisher Scientific (Pittsburgh, PA). Kimwipes EX-L paper was purchased from Kimberly-Clark (Roswell, GA).

2.2. Fungal strain and culture conditions

The organism used in the study was *Trichoderma atroviride* (formerly *T. harzianum* [8]) strain P1 (ATCC 74058). Dual-step cultures were used. In the first step, spore suspensions were inoculated at levels defined in the experimental design into 250-mL flasks containing 50 mL of potato dextrose broth. After 20 h at 25 °C on a rotary shaker (150 rpm), mycelia were collected on Miracloth, washed two times with sterile deionized water and transferred into 250-mL Erlenmeyer flasks containing 100 mL of minimal medium for the second step. Each flask corresponded to an experiment run. Minimal medium contained 1.6 g/L $MgSO_4$, 0.6 g/L $CaCl_2$, 20 mg/L $FeCl_3{\cdot}6H_2O$, 2.5 mg/L $FeSO_4{\cdot}7H_2O$, 1.6 mg/L $MnSO_4{\cdot}H_2O$, 1.0 mg/L $ZnSO_4{\cdot}7H_2O$, 1.0 mg/L $CoCl_2$, 0.04 mg/L $CuSO_4{\cdot}5H_2O$, and 0.013 mg/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ and was buffered with 90 mM KH_2PO_4, pH 6.2. Concentrations of glucose, ammonium ion, chitin, and scleroglucan in each flask were varied at levels defined in the experimental design. Glucose 10× stock solutions were autoclaved separately and the specified amount was added to each flask under aseptic conditions.