Biocatalysis and Agricultural Biotechnology 1 (2012) 140–146

Contents lists available at SciVerse ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

Use of response surface methodology to optimize production of pectinases by recombinant *Penicillium griseoroseum* T20

Daniel Bonoto Gonçalves [a], Janaina Aparecida Teixeira [b], Denise Mara Soares Bazzolli [b], Marisa Vieira de Queiroz [b], Elza Fernandes de Araújo [b,*]

[a] *Campus Centro-Oeste Dona Lindu, Universidade Federal de São João del-Rei, 35501-296 Divinópolis, MG, Brazil*
[b] *Departamento de Microbiologia/BIOAGRO, Universidade Federal de Viçosa, 36570-000 Viçosa, MG, Brazil*

ARTICLE INFO

Article history:
Received 10 September 2011
Accepted 20 September 2011
Available online 9 January 2012

Keywords:
Penicillium griseoroseum
Response Surface Methodology
Pectinase production
Pectin lyase
Polygalacturonase

ABSTRACT

Response Surface Methodology (RSM) has been the most utilized optimization method in recent years. Lately, many studies have successfully applied this technique to enzyme production processes of industrial interest. In this context, the production process of pectin lyase (PL) and polygalacturonase (PG) by the genetically modified strain *Penicillium griseoroseum* T20 was studied. RSM enabled determination of optimal cultivation conditions for PL and PG production. The highest production of PL was obtained after 87.7 h in medium containing sucrose at an initial concentration of 15.7 g/L, and in this condition, the model estimated a PL activity of 2428 U/mL. The highest PG production was obtained after 83.8 h, and in this condition, the model estimated a PG activity of 9465 U/mL. The production of PL and PG between the *Penicillium griseoroseum* T20 and *Penicillium griseoroseum* wild type strains was compared after optimization, and increases of more 400 times were observed. Although the RSM presents some limitations, which were addressed in this work, the results show that it was successfully utilized. This provides evidence for great potential of industrial application of the *Penicillium griseoroseum* T20 strain for the production of PL and PG.

1. Introduction

Pectinases constitute a heterogeneous group of enzymes that degrade pectin (Gummadi and Panda, 2003) These enzymes are especially important in the industrial sector and are utilized in various segments, such as the clarification of fruit juice and wine, manufacturing of hydrolyzed pectin products, extraction of oil from oleaginous seeds and pigments and the degumming of natural fibers (Kashyap et al., 2001). The degradation of the pectin molecule is done through a synergistic and coordinated action of various pectinolytic enzymes, including pectin methylesterase, polygalacturonases, pectate lyases and pectin lyases. The pectin lyase (E.C. 4.2.2.10) and polygalacturonase (EC. 3.2.1.15) enzymes are of great relevance for the process of depolymerization of pectin, acting in the cleavage of the α-1,4-glycosidic bonds of polygalacturonic and pectic acids, respectively (de Vries and Visser, 2001; Gummadi and Kumar, 2005). For this reason, these enzymes have been the targets of studies that aim at obtaining expression systems on a large scale, especially for utilizing filamentous fungi (Cardoso et al., 2008; Ribeiro, 2005).

Recently, a genetically modified super producer strain of pectin lyase (PL) and polygalacturonase (PG) denominated *P. griseoroseum* T20 was obtained in our laboratories (Teixeira et al., 2011). This strain presents great potential for industrial application, producing increases of up to 62 and 12 times in the production of PL and PG, respectively, compared to the *P. griseoroseum* wild type strain. In this way, efforts to increase the output of the enzyme production process without increasing the operational costs are now necessary. Statistical methods for optimization of processes represent a safe and reliable alternative in comparison with the classic methods, which are based on the study of only one independent variable at a time, while all the other factors are maintained at a fixed level.

Response Surface Methodology (RSM) is a statistical method that utilizes quantitative data based on adequate experimental planning in order to determine and simultaneously resolve multivariate equations. This method represents a set of statistics techniques that aim at executing experimental planning, building empirical models and evaluating the effect of independent variables on the desired variable response (Box and Wilson, 1951). RSM has been extensively utilized to optimize chemical and biochemical processes, such as production of enzymes (Barbosa et al., 2010; Bhattacharya et al., 2010; Hajji et al., 2008; Li et al., 2007), composition of cultivation media (Kunamneni and Singh,

* Corresponding author. Tel.: +55 31 3899 2973; fax: +55 31 3899 2573.
E-mail address: ezfa@ufv.br (E.F. Araújo).

1878-8181/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bcab.2011.09.002

2005), conditions of enzymatic hydrolysis (Shieh and Lai, 2000), parameters for the polymers synthesis and parameters for food processing (Ozer et al., 2004).

The goal of the present study was to apply RSM in the optimization of PL and PG production through the recombinant *P. griseoroseum* T20 strain. Second-degree polynomial equations were adjusted to each variable response and enabled us to reach the optimum region of PL and PG production in cultivation in a volume of 200 mL. The fermentation was scaled to 10.5 L of cultivation medium. The enzymatic activities were evaluated in this condition, which provided important information about the industrial application of the *P. griseoroseum* T20 strain as a producer of pectinases.

2. Materials and methods

2.1. Microorganisms and growth conditions

The *P. griseoroseum* T20 recombinant strain (Teixeira et al., 2011) produce high levels of pectin lyase (PL) and polygalacturonase (PG) simultaneously and possesses additional copies of the genes *plg*1 (PL) and *pgg*2 (PG), and both are under control of the strong and constitutive promoter of the gene *gpd* and the termination region of the *A. nidulans trp*C gene. This strain was obtained from the *P. griseoroseum* T105 (Cardoso et al., 2008), a previously obtained strain containing the *plg*1 gene, that was transformed with the plasmid pAN52pgg2, containing the gene encoding PG of *P. griseoroseum*. The *P. griseoroseum* wild type strain (CCT6421), isolated at the Federal University of Viçosa, the *P. griseoroseum* PG63 mutant strain (Pereira et al., 2004) and the recombinant strains *P. griseoroseum* T105 (Cardoso et al., 2008) and T146 (Ribeiro, 2005) were utilized as experiment controls. The strains were cultivated in potato-dextrose-agar (PDA) medium (OXOID) in Petri dishes and maintained at 25 °C for seven days. The inoculum was obtained from a conidial suspension (10^6 spores/mL) obtained from the surface of solid medium in a sterile solution of 0.2% Tween 80 (v/v).

2.2. Cultivation in submerged fermentation

The strains were cultivated either in submerged fermentation in 500 mL Erlenmeyer flasks with a working volume of 200 mL or in a bioreactor New Brunswick BioFlo IV® 20 L with a working volume of 10 L. The initial quantity of inoculum was of 10^5 and 10^6 conidia per mL of cultivation medium in either the Erlenmeyer flasks fermentations or in the bioreactor, respectively. The temperature and agitation were maintained constantly at 25 °C and 150 rpm, respectively. The initial pH of the cultivation medium was 6.8. The strains were cultivated in minimal medium composed of the following (in g/L): 6.98K_2HPO_4; 5.44 KH_2PO_4; 1.0 $(NH_4)_2SO_4$; 1.1 $MgSO_4 \cdot 7H_2O$ pH 6.8 and the carbon source. The cultivation medium containing the microorganisms was filtered in a sieve of 400 mesh/square inches (37 μm pore), the supernatant stored at −20 °C and the mycelium utilized to determine the dry mycelial mass.

The optimization of the conditions for cultivation of the *P. griseoroseum* T20 strain was carried out in a working volume of 200 mL. The concentrations of commercial sucrose utilized were 5, 10, 15, 20 and 30 g/L. The carbon source utilized for cultivation of the *P. griseoroseum* CCT6421 and PG63 strains was commercial sucrose at a concentration of 15 g/L along with yeast extract at 0.6 g/L. In order to obtain expression of the *pgg*2 and *plg*1 genes in *P. griseoroseum* wild type, the culture medium must contain sucrose as the carbon source and yeast extract (0.06%) because these genes are not expressed in culture medium containing only sucrose (Ribeiro, 2005; Bazzolli et al., 2008).

2.3. Experimental planning

RSM was utilized to optimize the conditions of PL and PG production by the *P. griseoroseum* T20 strain cultivated in 500 mL Erlenmeyer flasks and this was carried out with a working volume of 200 mL of cultivation medium containing commercial sucrose as the carbon source. A full factorial plan 5^2 was utilized. The independent variables studied were the concentration of the carbon source sucrose (X_1), at concentrations of 5, 10, 15, 20 and 30 g/L, and the cultivation time (X_2), at times of 24, 48, 72, 96 and 120 h, which resulted in 35 experimental units. The response variables were defined as the production of PL (Y_1) and of PG (Y_2). A point was defined at which $X_1 = 10$ g/L, and in this case three replications were carried out.

The following equation describes the regression model utilized in the factorial planning, including the interaction terms:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response variable, with Y_1 referring to PL production and Y_2 referring to PG production, β_0 is the intercept coefficient, β_i are the coefficients of the linear effects, β_{ii} are the coefficients of the quadratic effects, β_{ij} are the coefficients of interaction and x_i and x_j are the independent variables concentration of the carbon source and cultivation time, respectively.

The Student t test was utilized in order to determine the statistical significance of the regression coefficients. The Fisher test for the analysis of variation (ANOVA) was carried out on the experimental data in order to evaluate the statistical significance of the model. The regression analyses and the graphic construction were carried out with the utilization of the statistics software R (Version 2.8.1, 2008; Lucent Technologies).

2.4. Enzyme assay

Culture supernatants were utilized for dosage of the PL and PG activities, protease and cellulase activities, respectively.

PL activity was determined according to methods previously described (Albersheim and Killias, 1962). One PL activity unit was defined as the quantity of enzyme necessary to produce a nanomole of $\Delta^{4,5}$ galacturonide per minute, utilizing the coefficient of molar extinction 5550 mol^{-1} L cm^{-1} for the calculation. PG activity was determined through the methodology described by Miller (1959). One unit of PG activity was defined as the quantity of enzyme necessary to produce one micromole of galacturonic acid per minute. Protease activity was measured according to methods described (Rajmohan et al., 2002). One unit of proteolytic activity was defined as the increase of one unit at an absorbance of 366 nm per minute of the reaction under the described conditions. Cellulase activity was determined according to methods described (Tuohy et al., 2002). The quantification of the total protein grade in the culture supernatant was carried out through the Bradford method (Bradford, 1976), and mycelial mass production was quantified according to methods described (Calam, 1969).

2.5. Extracellular protein profile

Polyacrylamide gel electrophoresis (SDS-PAGE) was conducted utilizing a separation gel and a stacking gel containing 0.1% SDS and 12% and 4.5% acrylamide, respectively. The proteins were stained with a 0.1% solution of Coomassie brilliant blue R-250.