

## INOCULUM PADRONIZATION FOR THE PRODUCTION OF CUTINASE BY *FUSARIUM OXYSPORUM*

Tatiana Fontes Pio\*; Laura Priscila Fraga; Gabriela Alves Macedo

Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos, Universidade do Estado de Campinas,  
Campinas, SP, Brasil

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### ABSTRACT

Cutinase is a versatile enzyme showing several interesting properties for application in industrial processes. The widespread use of this enzyme depends on the development of an efficient and low-cost production system. One of the most important steps in a fermentation process is the standardization of the inoculum characteristics. In this study, the production of cutinase by *Fusarium oxysporum* showed a statistically significant relationship with both the inoculum size and the inoculum PDA pH. The greatest activities were 19.1 U/mL at PDA pH 7.0 and 22.72 U/mL using an aliquot of  $12.72 \times 10^7$  spores/mL. The macroscopic characteristics of the colonies of *Fusarium oxysporum* changed according to the variation of the medium pH, with the best results recorded in those colonies presenting a cotton white aspect.

**Key words:** *Fusarium oxysporum*; inoculum; cutinase; macroscopic characteristics; fermentation process.

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### INTRODUCTION

Cutinase is a versatile enzyme showing several interesting properties for application in industrial processes. In the last few years, several works have been published illustrating the importance of transesterification in areas like the pharmaceutical industry (7), foods (8,18), chemicals (6) and peptide synthesis (17), among others (3). An important aspect regarding the widespread use of cutinase is the development of an efficient and low-cost production system, which maximises the biosynthesis of the enzyme while simplifying its recovery from the cultivation medium (2).

The fermentation process involves an inoculum development step, which is the preparation of a population of microorganisms from a stock dormant culture to a state useful for inoculating a final production fermenter. The preparation of the inoculum is usually done at the laboratory and then transferred to the production plant. Therefore, it is important that what is transferred is as consistent as possible, in terms of size and quality, so that control of the fermentation plant be as automated as possible. Although the importance of inoculum

development has long been recognized in determining the productivity of industrial fermentations, it has been little investigated (21).

*Fusarium oxysporum* produces three types of spores, called microconidia, macroconidia, and chlamydospores. Microconidia are one or two celled, and represent the type of spore most abundantly and frequently produced by the fungus under all conditions. They are also the type of spore most frequently produced within the vessels of infected plants. Macroconidia are three to five celled, commonly found on the surface of plants killed by this pathogen. The macroconidia are produced in a specialized structure called a sporodochium. Chlamydospores are the third type of spore and are either one or two celled. They are thick-walled spores filled with lipidlike material that serves to carry the fungus when a suitable host is not available, and can remain dormant in soil and infect other hosts for as long as 30 years (1,11,12).

*Fusarium sp.* rapidly grows in potato-dextrose agar (PDA) medium and produces woolly to cottony, flat, spreading colonies. From the top, the color of the colony may be white, cream, tan, salmon, cinnamon, yellow, red, violet, pink, or purple. On the

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\*Corresponding Author. Mailing address: Food Science Department, Faculty of Food Engineering, Campinas State University, UNICAMP, P.O. Box 6121, CEP 13083-862, SP, Brasil. Phone: + 55 (19) 3521-2175; Fax +55 (19) 2121-7395. E-mail: tatipi@fea.unicamp.br

underside, it may be colorless, tan, red, dark purple, or brown. A sclerotium, which is the organized mass of hyphae that remains dormant during unfavorable conditions, may be observed macroscopically and is usually dark blue in color. On the other hand, sporodochium, the cushionlike mass of hyphae bearing macroconidia, is usually absent in culture. When present, it may be observed in cream to tan or orange color, except for *Fusarium solani*, which gives rise to blue-green or blue sporodochia (4,16,20).

The objective of the present work is to evaluate the influence of the inoculum size and the PDA medium pH on the production of cutinase by *F. oxysporum*. The authors also performed a qualitative analysis of the macroscopic characteristics of the colonies of *F. oxysporum*, grown in PDA in several pH values. The study of the influence of the inoculum size demanded an univariate analysis, due to its high degree of variability, which would introduce a great level of error in the multivariate experiments. Webb and Kamat (21), in a study of improving fermentation consistency through better inoculum preparation, obtained a range of variability of 1069% in the number of cells transferred from agar slants using conventional loop transfer. This variability was reduced to 208% using liquid transfer from slants previously inoculated by wire loop. Examination of typical inoculum development programmes for industrial fermentation shows that the initial stages invariably involve transfer of cells from solid cultures, generally made using a wire loop, being impossible to control accurately the number and condition of cells transferred in this way. It is quite likely that considerable variation is introduced at the very beginning of the process (13,19).

## MATERIAL AND METHODS

### Microorganism

The authors employed a strain of *F. oxysporum* previously selected as the best producer of cutinase, from a screening of 400 strains of fungi from samples of plants, fruits, leaves and bark of trees collected in the field at different areas of São Paulo State (9). *F. oxysporum* was stocked in PDA at 4°C.

### Cutinase assay

Cutinase activities were assayed in the enzyme-containing supernatant with p-nitrophenyl-butyrate (pNPB) as substrate, determined spectrophotometrically at 405 nm after 15 minutes (2). An aliquot (0.070 mL) of the culture medium broth supernatant was added to 3.430 mL of a reaction mixture composed of 1.12 mM of pNPB, 50 mM phosphate buffer pH 7.2, 0.2% (N/P) Triton X-100 and 0.43 M tetrahydrofuran. One cutinase unit (U) is defined as the amount of cutinase required to convert one micromole of p-nitrophenyl in one minute under the specified conditions. pNPB was purchased from Sigma-Aldrich Brazil Co. (São Paulo, SP, BR).

### Influence of the PDA slant pH of the inoculum in the production of cutinase in liquid mineral medium

The inoculum was grown on PDA slants at pH 4.0, 7.0 and 10.0. In order to adjust the pH value the authors added 1.0 N NaOH or HCl as necessary. After 72 hours at 30°C, aliquots of 5 mL sterile distilled water were added to each slant. Aliquots containing  $6.2 \times 10^7$  spores of the inoculum were added to the liquid mineral medium. This medium was defined in an optimization study by Pio and Macedo (14), and has the following composition: 0.06% NaNO<sub>3</sub>, 0.06% K<sub>2</sub>HPO<sub>4</sub>, 0.02% MgSO<sub>4</sub>, 0.02% KCl and 0.01% FeSO<sub>4</sub>·7H<sub>2</sub>O, pH 7.2. The cutinolytic activity was measured as described on item 2.2, after 48 hours of fermentation at 100 rpm and 30°C. All data were analyzed using the software "Statistica for Windows" (Microsoft, version 5.0, 1995). The mean values were compared using the Tukey test, at a confidence interval of 95% and significance level of  $p \leq 0.05$ .

### Effect of inoculum size

The production of cutinase by *F. oxysporum* was measured employing inoculum spores concentrations of  $12.72 \times 10^7$ ,  $6.20 \times 10^7$  and  $3.75 \times 10^7$  spores/mL. The inoculum was prepared through the addition of sterile distilled water to PDA slants at pH 7.0, after 72 hours of growth at 30°C. In order to adjust the pH value the authors added 1.0 N NaOH as necessary. Aliquots containing 1 mL of the inoculum were added to 50 mL conical flasks containing 20 mL of liquid mineral medium, and the flasks maintained at 100 rpm and 30°C for 48 hours before readings. These conditions were determined after an optimization study carried out by Pio and Macedo (not published). All the tests were carried out twice.

### Effect of the PDA medium pH on the micellar growth of *F. oxysporum*

In an attempt to correlate the cutinolytic activity with the macroscopic appearance of the colonies, Petri plates containing 20 mL PDA at the pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, were inoculated in the center of the plate. In order to adjust the pH value the authors added 1.0 N NaOH or HCl as necessary. For the plates with pH values between 4.0 and 7.0, the pH was adjusted before sterilization, and became stable. However, the plates with pH values between 8.0 and 10.0, further adjustment were required after sterilization. All the tests were performed twice. The macroscopic characteristics were observed at 24, 48, 72, 96, 120, 144 and 168 hours of incubation at 30°C. The color and size of the colony were observed in a qualitative way.

## RESULTS AND DISCUSSION

### Influence of the PDA slant pH of the inoculum in the production of cutinase in liquid mineral medium

The values for cutinolytic activity after 48 hours of fermentation were 9.0, 19.1 and 15.6 U/mL at pH values of 4.0,

7.0 and 10.0, respectively. The results were statistically different from each other, as shown by the Tukey test with 95% confidence interval and level of significance of  $p < 0.05$ .

**Influence of inoculum concentration on the production of cutinase**

The relationship between inoculum concentration and cutinase activity is described on Table 1. The best production of cutinase was 22.7 U/mL, with a standard deviation of 0.8 U/mL, obtained with an aliquot of  $12.72 \times 10^7$  spores/mL. It was not possible to increase the concentration of the inoculum further due to limitations of the method. There was a significant difference between the three dilutions in the production of cutinase after 48 hours, demonstrated through the Tukey test, at a confidence interval of 95% and a significance level of  $p \leq 0.05$ . The importance of the inoculum size has long been recognized to determine the productivity of industrial fermentations, being investigated quantitatively by Webb and Kamat (21), using *Saccharomyces cerevisiae*.

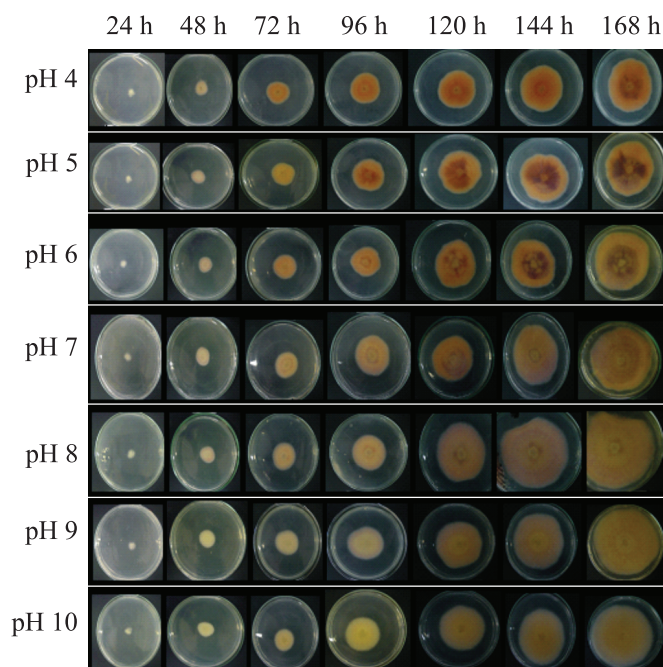
**Effect of PDA medium pH on the micellar growth of *F. oxysporum***

The behavior of the *F. oxysporum* colonies as a function of the growth medium pH and the incubation time is described on Fig. 1. The macroscopic morphology of the colonies was cotton white, irrespective of the pH value at 24 hours. At 48 hours, there was the presence of salmon hue at pH 4.0, while the greatest colony diameter was observed at pH 7.0. At 72 hours, there was a turn into salmon at pH values of 5.0 and 6.0. At pH 7.0 and 8.0, there was the formation of a salmon halo. There was no change in color at pH of 9.0 and 10.0. At 96 hours, there was the formation of a purple blot at the center of the colony at pH 5.0. At 120 hours, there was an increase in the size of the purple blot at pH 5.0, and the formation of a purple blot at the center of the colony at pH 6.0, 7.0 and 8.0, and the formation of a salmon halo at the periphery of the colonies at pH 9.0 and 10.0. At 144 hours, there was an increase in the size of the purple blot at pH 5.0 and 6.0. At 168 hours, there was the formation of a purple blot at the center of the colony at pH 4.0.

According to Seifert (15), *F. oxysporum* colony pigmentation can vary in different pH (15). PDA made according to the

**Table 1.** Effect of inoculum size on cutinase production by *F. oxysporum*, submitted to analysis by the Tukey test, at a 95% confidence interval.

Spores ( $\times 10^7$ /mL)	Cutinase activity (U/mL)	S.D. (U/mL)
12.72	22.7 <sub>A</sub>	0.8
6.2	18.4 <sub>B</sub>	0.1
3.75	6.0 <sub>C</sub>	0.4



**Figure 1.** Effect of PDA pH on the growth of *F. oxysporum* colonies at 24, 48, 72, 96, 120, 144 and 168 hours after inoculation.

specifications of Nelson *et al.* (10), is a valuable medium used principally for noting gross morphological appearances and colony coloration. Because of its high available carbohydrate content, PDA generally emphasizes growth in the detriment of sporulation. Cultures grown on this medium sporulate poorly, frequently taking more than a month to do so. The conidia produced are often misshapen and atypical. Consequently, with few exceptions, PDA cultures are not used for microscopic observation (11). Morphology of spores on PDA or from the host is usually very variable and not reliable (5).

**CONCLUSION**

In this study, there was a statistically significant relationship between cutinase activity and both inoculum concentration and PDA medium pH values.

The inoculum PDA pH had a statistically significant influence in the production of cutinase by *F. oxysporum* in liquid media at 48 hours of fermentation, with the best result being 19.1 U/mL at pH 7.0.

The best production of cutinase was 22.7 U/mL, with a standard deviation of 0.8 U/mL, obtained with an aliquot of  $12.72 \times 10^7$  spores/mL. It was not possible to increase the concentration of the inoculum further due to limitations of the method.

The macroscopic characteristics of the colonies of *F. oxysporum* changed with the variation of the medium pH, possible due to metabolic alterations or to the production of

spores. The cotton white aspect was associated with higher levels of cutinase production. Further studies are needed to clarify the meaning of these macroscopic changes, involving the microscopic registration of the organism.

It is possible to increase the production of cutinase by *F. oxysporum* through the optimization of the inoculum concentration and PDA medium pH. This is an important issue when addressing the planning of industrial fermentation processes.

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### RESUMO

#### Padronização do inóculo para a produção de cutinase por *Fusarium oxysporum*

Cutinase é uma enzima versátil, que apresenta propriedades interessantes para aplicação em processos industriais. O uso desta enzima em larga escala depende do desenvolvimento de um sistema de produção eficiente e de baixo custo. Uma das etapas mais importantes em um processo de fermentação é a padronização do inóculo. Neste estudo, houve uma associação estatisticamente significativa entre a produção de cutinase por *Fusarium oxysporum* e tamanho do inóculo e pH do meio PDA. As maiores atividades de cutinase foram 19,1 U/mL em PDA com pH 7,0 e 22,72 U/mL empregando um inóculo de  $12,72 \times 10^7$  esporos/mL. As características macroscópicas das colônias de *Fusarium oxysporum* mostraram alterações em função do pH do meio, com as maiores atividades sendo registradas em presença de colônias brancas com aspecto cotonoso.

**Palavras-chave:** *Fusarium oxysporum*; inóculo; cutinase, características macroscópicas; processo de fermentação.

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