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Cutinase production by *Fusarium oxysporum* in liquid medium using central composite design

Tatiana Fontes Pio · Gabriela Alves Macedo

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Abstract The objective of the present study was to measure the production of cutinase by *Fusarium oxysporum* in the presence of several carbon and nitrogen sources (glycides, fatty acids and oils, and several organic and inorganic nitrogen sources), trying to find a cost-effective substitute for cutin in the culture medium as an inducer of cutinase production. The results were evaluated by the Tukey test, and flaxseed oil was found to give the best results as a cutinase inducer. The authors optimized the composition of the growth medium employing response surface methodology. The experimental results were fitted to a second-order polynomial model at a 95% level of significance ($p < 0.05$). The greatest cutinolytic activity was obtained in a liquid mineral medium supplemented with flaxseed oil, showing an increase in enzymatic activity from 11 to 22.68 U/mL after 48 h of fermentation. A CCD study of the fermentation conditions was carried out, and the best production of cutinase was registered with the use of 30 °C and 100 rpm. These results support the use of flaxseed oil as a substitute for cutin, which is difficult and expensive to obtain, for the production of cutinase in a larger scale.

Keywords Cutinase · *Fusarium oxysporum* · Liquid medium · Inoculum · Central composite design

T. F. Pio (✉) · G. A. Macedo
Food Science Department, Faculty of Food Engineering, Campinas State University (UNICAMP), P.O. Box 6121, CEP 13083-862 Campinas, SP, Brazil
e-mail: tatipi@fea.unicamp.br

Introduction

Cutinases are enzymes excreted by phytopathogenic fungi and responsible for the in vivo hydrolysis of cutin, a biopolymer that covers and protects plants against pathogenic microorganisms [1].

Cutin is a non-soluble biopolyester which makes up the structural component of the plant cuticle, consisting of the following fatty acids ω-hydroxy fatty acids, dihydroxy palmitic acid, 18-hydroxy-9,10,-epoxy C18 saturated and Δ12 unsaturated acids, and 9,10,18-trihydroxy C18 saturated and Δ12 monounsaturated acids. The precise composition of cutin depends on the species, but as a rule, the cutin of fast-growing plants seems to consist mainly of 16 carbon acids, especially dihydroxypalmitate, whereas the slow-growing plants show both 16- and 18 carbon acids. Cutinase enzymatically removes all types of monomer from the polymer [7, 13, 21].

Cutinase can be classified as EC 3.1.1.3, the recommended name being triacylglycerol lipase, and the systematic name triacylglycerol acyl hydrolase. The same source also classifies it as 3.1.1.74, the recommended name being cutinase and systematic name cutin hydrolase. It is first considered as an esterase that hydrolyzes cutin, but can also work as and be classified as a lipase [12].

This relatively small globular protein, $45 \times 30 \times 30$ Å in size, with 197 residues and a molecular mass of 22 kDa, has had its 3-D structure resolved at 1.0 Å. Cutinase possesses an α/β fold common to the α/β hydrolase family, with a central β-sheet of five parallel strands flanked by two α-helices on one side and three on the other. Along with the characteristic secondary structure fold it possesses conserved sequences of residues of which the most noteworthy is the active site stretch of amino acids containing the catalytic nucleophile Serine 120: Gly-His/Tyr-Ser-X-

Gly. This consensus sequence is common to many lipases and the catalytic triad Ser 120, His 188 and Asp 175 includes cutinase in the serine hydrolase class [6, 9].

Cutinase is a versatile enzyme showing several interesting properties for application in industrial processes. An enzymatic preparation containing cutinase has been developed to increase the pharmacological effect of agricultural chemicals and it has also been applied as a lipolytic enzyme for fat removal in laundry and dishwashing detergents. Degradation of plastics such as the synthetic polyester polycaprolactone, into water-soluble products, has been achieved using cutinase [17].

Other potential uses include the hydrolysis of milk fat in the dairy industry, applications in the oleochemistry industry, in the synthesis of structured triglycerides and in polymers and surfactants for personal-care products, and in pharmaceuticals and agrochemicals containing one or more chiral centers [17].

In the last few years, several works have been published illustrating the importance of transesterification in areas like the pharmaceutical industry [11], foods [20, 15], chemicals [10] and peptide synthesis [19], among others [3].

In 2005, Macedo and Pio [16] isolated 400 strains of fungi from soil and plants collected in different regions of Brazil. The objective of this work was to isolate microorganisms for a pre-selection of fungi showing esterase producing ability using a fast test in Petri dishes. The selected strains were then inoculated in media containing cutin, and the lipolytic and cutinolytic activities of the supernatant determined in order to differentiate lipase producers from cutinase producers. The fungal strain selected as the best cutinase producer was identified as *Fusarium oxysporium.*

The traditional one-at-a-time optimization strategy is simple and useful in screening procedures, and the individual effects of medium components can be seen on a graph with no need to revert to more sophisticated statistical analyses. Unfortunately, this simple method frequently fails to locate the optimal response region, because the joint effects of factors on the response are not taken into account. It has been reported that the complexities and uncertainties associated with large-scale fermentations usually arise from a lack of knowledge of the sophisticated interactions amongst the various factors acting during fermentation [6].

Statistical experimental designs provide an efficient approach to optimization. The fractional factorial design (FFD) is especially suitable in accounting for the interactions and identifying the more significant components in a medium formula. A combination of factors generating a certain optimal response can be identified from the use of a factorial design and response surface methodology [14].

Response surface methodology (RSM) is a powerful technique for testing multiple process variables, because fewer experimental trials are needed as compared to studying one variable at a time. Also, significant interactions between the variables can be identified and quantified by this technique.

An important aspect of the commercialization of cutinase is the development of an efficient and low-cost production system, which maximises the biosynthesis of the heterologous protein while simplifying its recovery from the cultivation medium [2].

In this setting, one of the main drawbacks is the expensive, time-consuming and low yield process of producing cutin. The present work aimed at finding an alternative to cutin as an inducer of cutinase production. Ideally, this substance should be easily obtainable and result in a high process yield. In order to further increase the viability of the process, the authors carried out an optimization study with respect to the medium composition and the inoculum.

Materials and methods

Strain and cultivation condition

Fusarium oxysporum, obtained from soil and plants collected in different regions of Brazil, was maintained on potato dextrose agar (PDA) slants and stored at 4° C. The strain was replicated in PDA and incubated at 30 °C for 72 h and the inoculum prepared by adding 5 mL of distilled water to each slant. Aliquots containing 1 mL of this spore suspension were added to 20 mL of the mineral medium (0.06% $NaNO_3$, 0.06% K_2HPO_4, 0.02% $MgSO_4$, 0.02% KCl, 0.01% $FeSO_4{\cdot}7H_2O$, pH 7.2) in the presence of a cutinase inducer (cutin or different carbon or nitrogen sources), in 50 mL unbaffled Erlenmeyer flasks, and cultivated for 2 days at 30 °C and 100 rpm in a rotary shaker. After this, the cultures were centrifuged at 10 °C and 10,000 rpm (9,050 × *g*) for 15 min, and the supernatants collected for the analysis of cutinase activity.

Cutinase assay

Cutinase activity was determined spectrophotometrically following the hydrolysis of *p*-nitrophenylbutyrate (*p*NPB) at 405 nm. An aliquot (0.070 mL) of the culture supernatant was added to 3.43 mL of a reaction mixture with the following composition 1.12 mM *p*NPB dissolved in 50 mM phosphate buffer, pH 7.2, also containing 0.2% (N/P) Triton X-100 and 0.43 M tetrahydrofuran. The reaction was monitored for 15 min against a blank solution. One unit of

cutinolytic activity was defined as the amount of cutinase required to release one micromole of *p*-nitrophenyl in 1 min under the specified conditions. *p*NPB was purchased from Sigma-Aldrich Brasil Co. (São Paulo, SP, BR).

Screening of carbon and nitrogen sources

In order to select the best nitrogen and carbon sources to replace apple cutin in the production of cutinase, some sources were investigated using the univariable strategy. The following nitrogen sources were investigated and their concentration was fixed at a level of 0.06% urea, ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate and sodium nitrite.

Several carbon sources were tested, using concentrations of 0.5 and 1.0% glycides (glucose, fructose, maltose, galactose, lactose, sucrose), some fatty acids (lauric, stearic, oleic and palmitic) and added oils (flaxseed, rice, peanut, maize and olive).

In the investigation of nitrogen sources, growth was carried out in a medium without a carbon source, containing 0.06% $NaNO_3$, 0.06% K_2HPO_4, 0.02% $MgSO_4$, 0.02% KCl and 0.01% $FeSO_4{\cdot}7H_2O$, pH 7.2. The tests were done in duplicate and the average values were then submitted to the Tukey test, at a 95% confidence interval.

Optimization procedure and experimental design

Screening of factors significantly affecting cutinase production

A $2^{(6-2)}$ fractional factorial design was employed to determine the key ingredients significantly affecting cutinase production. The six nutrient factors in the medium were flaxseed oil, K_2HPO_4, $MgSO_4$, $NaNO_3$, KCl and $FeSO_4{\cdot}7H_2O$. Each factor was examined at a high level (coded + 1) and a low level (coded – 1). The central points were the trials with the basal level conditions (coded 0). Table 2 shows the variables and levels in detail. A ¼ fraction of the full factorial design was adopted, resulting in a total of 20 tests, including 16 combinations and 4 repetitions at the central point. Each test was performed once, and the enzyme activity was measured after 48 h of fermentation.

Response surface methodology

The medium components that significantly affected cutinase production were optimized using a CCD design. The variables were coded according to the following equation

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where x_i is the coded variable for a nutrient factor, X_i is the natural variable of the nutrient factor, X_0 is the value of the natural variable at the centre point and ΔX_i is the step change value. The variables and levels are shown in Table 3.

The results were fitted to a second-order polynomial function. The Student's *t* test was used to assess the statistical significance of the regression coefficients, and the analysis of variances (ANOVA) was then performed using only the statistically significant experimental terms. The response model was expressed using the coded variables, taking into account only these terms.

Experimental validation of the optimized conditions

The validation of the response surface was carried out through five repetitions of the optimal conditions.

Evaluation of fermentation parameters for the optimization of cutinase production in shaken flasks through CCD

A CCD strategy was employed in order to measure the effect of temperature and agitation on the production of cutinase after 48 h of fermentation. The CCD was composed by 8 tests plus 6 central points, in a total of 14 tests. The variables and levels are shown in Table 3.

Effect of inoculum size

The preparation of the inoculum was standardized employing aliquots of 2.5; 5.0 and 10 mL of sterile distilled water for each PDA slant inoculated with *F. oxysporum*, after 72 h of growth. This length of time was based on previous studies.

Aliquots containing 1 mL of the inoculum were added to 50 mL conical flasks containing 20 mL of liquid mineral medium, and maintained at 100 rpm and 30°C for 48 h before readings.

Results and discussion

Screening for carbon and nitrogen sources

The results of the various carbon and nitrogen sources effect on cutinase activity are described in Table 1. Data

Table 1 Averages for the production of cutinase (U/mL) after 48 h of fermentation, submitted to analysis by the Tukey test, at a 95% confidence interval

Independent variables (%)	Cutinase (U/mL) ± SD
Cutin 0.5	$11.20 \pm 0.19_C$
Flaxseed oil (0.5, 1.0)	$13.40 \pm 0.10_B$; $15.62 \pm .20_A$
Olive oil (0.5, 1.0)	$3.00 \pm 0.52_B$; $1.70 \pm 0.27_F$
Mamona oil (0.5, 1.0)	$1.40 \pm 0.18_G$; $1.00 \pm 0.05_G$
Soy oil (0.5, 1.0)	$3.40 \pm 0.20_E$; $2.42 \pm 0.49_F$
Sunflower oil (0.5, 1.0)	$0.54 \pm 0.56_G$; $1.70 \pm 0.1_F$
Palm oil (0.5, 1.0)	$6.10 \pm 0.46_D$; $5.40 \pm 0.37_D$
Peanut oil 0.5	$4.40 \pm 0.16_E$
Rice oil 0.5	$3.00 \pm 0.52_F$
Corn oil 0.5	$3.40 \pm 0.03_E$
Lauric acid 0.5	$0.74 \pm 0.36_G$
Stearic acid 0.5	$4.07 \pm 0.38_E$
Oleic acid 0.5	$2.75 \pm 0.17_F$
Palmitic acid 0.5	$5.34 \pm 0.45_D$
Yeast extract 0.06	$4.08 \pm 0.18_A$
Peptone 0.06	$2.22 \pm 0.17_B$
Ammonium sulphate 0.06	$0.18 \pm 0.01_C$
Urea 0.06	$0.36 \pm 0.05_C$
Ammonium nitrate 0.06	$0.40 \pm 0.02_C$
Potassium nitrate 0.06	$0.16 \pm 0.03_C$
Sodium nitrite 0.06	$0.22 \pm 0.03_C$
Sodium nitrate 0.06	$0.46 \pm 0.05_C$

The results of the Tukey test are displayed as letters indicating statistically different values

regarding the glycides group are not shown, because the cutinolytic activity was less than 0.5 U/mL for this group as a whole.

Some of the nitrogen sources showed a positive effect on cutinolytic activity when used without a carbon source. The organic nitrogen sources had the best results. The highest value for cutinolytic activity was 4.21 U/mL, obtained in the presence of yeast extract after 48 h of fermentation.

In 2007, Rispoli and Shah [18] studied the production of cutinase from *Collerotrichum lindemuthianum*, and found that yeast extract had a strong positive influence on the yield of the process.

The addition of 0.1% glucose to the medium containing a nitrogen source reduced cutinase activity to less than 0.5 U/mL. This effect was registered with all nitrogen sources tested. In 1996, Christakopoulos et al. [4] found similar results for the production of xylanase by *F. oxysporum*, with glucose inhibiting the cutinase activity for the majority of the nitrogen sources tested.

With respect to the carbon source, the best cutinolytic activity after 48 hours of fermentation was 15.62 U/mL, obtained using 1.0% flaxseed oil, followed by 6.10 U/mL using 0.5% palm oil,. There are very few publications dedicated to optimization of production of cutinase with regard to the growth medium conditions. Fett et al., 1999 [8], in their report on the production of cutinase by *Thermomonospora fusca*, found that the presence of glucose in the growth media had a negative effect on the production of cutinase, and that the fatty acids and oils tested resulted in better activity. Rispoli and Shah, 2007 [18], found that glucose had a weak influence on cutinase production by *Collerotrichum lindemuthianum*.

Screening of the factors significantly affecting cutinase production

The first strategy was a 2^{6-2} fractional design plus 4 central points, resulting in a total of 20 assays, which were analysed after 48 hours of fermentation. Using such a strategy, it was possible to analyze the main effects and indications of the independent variables for inclusion in the next test, and the new range of values for each variable.

The experimental design and the results of the FFD observations are shown in Table 2. Cutinase production varied greatly with the different combinations of the media components, from less than 0.5 to 10.00 U/mL.

From an analysis of the effects, using the pure error of the variables measured for enzymatic activity after 48 hours as the end point, the authors found that only K_2HPO_4 and $MgSO_4$ had not statistically significant effects, admitting a confidence interval of ±90%. Rispoli and Shah, 2007 [18], found that K_2HPO_4 had a positive influence on the production of cutinase by C*ollerotrichum lindemuthianum*, but $MgSO_4$ had a minimal impact on enzyme production.

The tests revealed that flaxseed oil, $NaNO_3$, KCl and $FeSO_4{\cdot}7H_2O$ all had an impact on cutinase activity. Increments in the concentrations of flaxseed oil and $FeSO_4{\cdot}7H_2O$ resulted in a decrease in cutinase activity, while increments in $NaNO_3$ and KCl lead to a higher cutinolytic activity.

Experimental design for medium composition optimization

The experimental design and results are shown in Table 3. The quadratic model calculated for maximum cutinase activity, after eliminating the statistically insignificant terms ($p > 0.05$), was

Table 2 Study of the effects of the variables, according to fractional factorial design, analysed after 48 h of fermentation

Run	Flaxseed oil (%)	$NaNO_3$ (%)	K_2HPO_4 (%)	$MgSO_4$ (%)	KCl (%)	$FeSO_4{\cdot}7H_2O$ (%)	Cutinase U/mL
1	–1 (0.5)	–1 (0)	–1 (0)	–1 (0)	–1 (0)	–1 (0)	1.00
2	+1 (1.5)	–1 (0)	–1 (0)	–1 (0)	+1 (0.04)	–1 (0)	1.07
3	–1 (0.5)	+1 (0.12)	–1 (0)	–1 (0)	+1 (0.04)	+1 (0.02)	1.00
4	+1 (1.5)	+1 (0.12)	–1 (0)	–1 (0)	–1 (0)	+1 (0.02)	1.93
5	–1 (0.5)	–1 (0)	+1 (0.12)	–1 (0)	+1 (0.04)	+1 (0.02)	0.50
6	+1 (1.5)	–1 (0)	+1 (0.12)	–1 (0)	–1 (0)	+1 (0.02)	1.04
7	–1 (0.5)	+1 (0.12)	+1 (0.12)	–1 (0)	–1 (0)	–1 (0)	4.05
8	+1 (1.5)	+1 (0.12)	+1 (0.12)	–1 (0)	+1 (0.04)	–1 (0)	2.17
9	–1 (0.5)	–1 (0)	–1 (0)	+1 (0.04)	–1 (0)	+1 (0.02)	<0.50
10	+1 (1.5)	–1 (0)	–1 (0)	+1 (0.04)	+1 (0.04)	+1 (0.02)	<0.50
11	–1 (0.5)	+1 (0.12)	–1 (0)	+1 (0.04)	+1 (0.04)	–1 (0)	5.50
12	+1 (1.5)	+1 (0.12)	–1 (0)	+1 (0.04)	–1 (0)	–1 (0)	0.69
13	–1 (0.5)	–1 (0)	+1 (0.12)	+1 (0.04)	+1 (0.04)	–1 (0)	1.04
14	+1 (1.5)	–1 (0)	+1 (0.12)	+1 (0.04)	–1 (0)	–1 (0)	0.57
15	–1 (0.5)	+1 (0.12)	+1 (0.12)	+1 (0.04)	–1 (0)	+1 (0.02)	2.09
16	+1 (1.5)	+1 (0.12)	+1 (0.12)	+1 (0.04)	+1 (0.04)	+1 (0.02)	2.30
17	0 (1.0)	0 (0.06)	0 (0.06)	0 (0.02)	0 (0.02)	0 (0.01)	10.00
18	0 (1.0)	0 (0.06)	0 (0.06)	0 (0.02)	0 (0.02)	0 (0.01)	9.80
19	0 (1.0)	0 (0.06)	0 (0.06)	0 (0.02)	0 (0.02)	0 (0.01)	9.50
20	0 (1.0)	0 (0.06)	0 (0.06)	0 (0.02)	0 (0.02)	0 (0.01)	10.00

$$Y = 10.73 - 0.66X_1 - 0.79X_1^2 - 0.70X_2 - 0.94X_2^2 - 0.84X_3 - 1.08X_3^2 - 1.56X_4 - 0.74X_4^2 - 0.61X_5 - 0.67X_5^2 - 1.17X_6 - 0.96X_6^2$$

In the equation, Y represents cutinase activity (U/mL); X_1, X_2, X_3, X_4, X_5 and X_6 represent, respectively, the concentrations of flaxseed oil, K_2HPO_4, $MgSO_4$, $NaNO_3$, KCl and $FeSO_4{\cdot}7H_2O$ (% volume).

The analysis of variance (ANOVA), reproduced in Table 4, shows that the model was significant. The Fisher F test ($F_{9.17} = 8.7 > F_{c\ 24;\ 56;\ 0.05} = 1.70$) was 5 to 6 times higher than F_t, demonstrating that this regression was statistically significant at the 95% confidence level. In addition, the R^2 (multiple correlation coefficient) of the regression equation obtained was 0.81 (a value > 0.75 indicates aptness of the model), suggesting that the proposed experimental design was suitable for the simulation of cutinase production by *F. oxysporum.*

The response surface and contour diagram for cutinase activity, considering flaxseed oil and $NaNO_3$, were chosen among the possible combinations, as representative of each selected rotational speed, in order to visualize the simultaneous effect of flaxseed oil, K_2HPO_4, $MgSO_4$, $NaNO_3$, KCl and $FeSO_4{\cdot}7H_2O$ on the cutinase production pattern (Fig. 1). The optimal concentrations of the six key ingredients were basically at the lowest levels (coded – 1) 1.0% flaxseed oil, 0.06% K_2HPO_4, 0.02% $MgSO_4$, 0.06% $NaNO_3$, 0.02% KCl and 0.01% $FeSO_4{\cdot}7H_2O$.

Experimental validation of the optimized conditions of the culture medium

All the tests were carried out at 30 °C and 100 rpm, and cutinase production measured after 48 h of fermentation. The mean value obtained from five repetitions was 17.52 U/mL, with a standard deviation of ±6 %. These results were in agreement with those obtained using CCD, and were considered by the authors as a validation of the optimal composition of the growth medium used in this study. Similar data were obtained by Christakopoulos et al. [5], in a study about the production of esterase by *F. oxysporum.* Under optimal conditions, these authors reported a yield of 7.3 U/mL in a liquid growth medium and 19.4 U/g in a solid medium.

Experimental design for fermentation conditions optimization

The experimental design and results are shown in Table 5. The best production of cutinase was 16.44 U/mL, obtained using 30° C and 100 rpm. The analysis of variance

Table 3 Optimization of the medium composition for the production of cutinase (U/mL), according to a central composite design (CCD), analysed after 48 h of fermentation

Run	Flaxseed oil (%)	$NaNO_3$ (%)	K_2HPO_4 (%)	$MgSO_4$ (%)	KCl (%)	$FeSO_4 \cdot 7H_2O$ (%)	Activity U/mL
1	–1 (1.0)	–1 (0.06)	–1 (0.06)	–1 (0.02)	–1 (0.02)	–1 (0.01)	18.84
2	+1 (2.0)	–1 (0.06)	–1 (0.06)	–1 (0.02)	–1 (0.02)	–1 (0.01)	13.14
3	–1 (1.0)	+1 (0.12)	–1 (0.06)	–1 (0.02)	–1 (0.02)	–1 (0.01)	12.85
4	+1 (2.0)	+1 (0.12)	–1 (0.06)	–1 (0.02)	–1 (0.02)	–1 (0.01)	9.26
5	–1 (1.0)	–1 (0.06)	+1 (0.12)	–1 (0.02)	–1 (0.02)	–1 (0.01)	14.83
6	+1 (2.0)	–1 (0.06)	+1 (0.12)	–1 (0.02)	–1 (0.02)	–1 (0.01)	12.52
7	–1 (1.0)	+1 (0.12)	+1 (0.12)	–1 (0.02)	–1 (0.02)	–1 (0.01)	6.53
8	+1 (2.0)	+1 (0.12)	+1 (0.12)	–1 (0.02)	–1 (0.02)	–1 (0.01)	4.82
9	–1 (1.0)	–1 (0.06)	–1 (0.06)	+1 (0.04)	–1 (0.02)	–1 (0.01)	6.29
10	+1 (2.0)	–1 (0.06)	–1 (0.06)	+1 (0.04)	–1 (0.02)	–1 (0.01)	4.63
11	–1 (1.0)	+1 (0.12)	–1 (0.06)	+1 (0.04)	–1 (0.02)	–1 (0.01)	4,13
12	+1 (2.0)	+1 (0.12)	–1 (0.06)	+1 (0.04)	–1 (0.02)	–1 (0.01)	3.45
13	–1 (1.0)	–1 (0.06)	+1 (0.12)	+1 (0.04)	–1 (0.02)	–1 (0.01)	6.39
14	+1 (2.0)	–1 (0.06)	+1 (0.12)	+1 (0.04)	–1 (0.02)	–1 (0.01)	3.70
15	–1 (1.0)	+1 (0.12)	+1 (0.12)	+1 (0.04)	–1 (0.02)	–1 (0.01)	5.65
16	+1 (2.0)	+1 (0.12)	+1 (0.12)	+1 (0.04)	–1 (0.02)	–1 (0.01)	2.90
17	–1 (1.0)	–1 (0.06)	–1 (0.06)	–1 (0.02)	+1 (0.04)	–1 (0.01)	16.77
18	+1 (2.0)	–1 (0.06)	–1 (0.06)	–1 (0.02)	+1 (0.04)	–1 (0.01)	14.40
19	–1 (1.0)	+1 (0.12)	–1 (0.06)	–1 (0.02)	+1 (0.04)	–1 (0.01)	10.71
20	+1 (2.0)	+1 (0.12)	–1 (0.06)	–1 (0.02)	+1 (0.04)	–1 (0.01)	10.24
21	–1 (1.0)	–1 (0.06)	+1 (0.12)	–1 (0.02)	+1 (0.04)	–1 (0.01)	6.73
22	+1 (2.0)	–1 (0.06)	+1 (0.12)	–1 (0.02)	+1 (0.04)	–1 (0.01)	4.70
23	–1 (1.0)	+1 (0.12)	+1 (0.12)	–1 (0.02)	+1 (0.04)	–1 (0.01)	4.83
24	+1 (2.0)	+1 (0.12)	+1 (0.12)	–1 (0.02)	+1 (0.04)	–1 (0.01)	4.20
25	–1 (1.0)	–1 (0.06)	–1 (0.06)	+1 (0.04)	+1 (0.04)	–1 (0.01)	7.75
26	+1 (2.0)	–1 (0.06)	–1 (0.06)	+1 (0.04)	+1 (0.04)	–1 (0.01)	3.52
27	–1 (1.0)	+1 (0.12)	–1 (0.06)	+1 (0.04)	+1 (0.04)	–1 (0.01)	4.31
28	+1 (2.0)	+1 (0.12)	–1 (0.06)	+1 (0.04)	+1 (0.04)	–1 (0.01)	2.67
29	–1 (1.0)	–1 (0.06)	+1 (0.12)	+1 (0.04)	+1 (0.04)	–1 (0.01)	5.83
30	+1 (2.0)	–1 (0.06)	+1 (0.12)	+1 (0.04)	+1 (0.04)	–1 (0.01)	2.90
31	–1 (1.0)	+1 (0.12)	+1 (0.12)	+1 (0.04)	+1 (0.04)	–1 (0.01)	3.48
32	+1 (2.0)	+1 (0.12)	+1 (0.12)	+1 (0.04)	+1 (0.04)	–1 (0.01)	1.76
33	–1 (1.0)	–1 (0.06)	–1 (0.06)	–1 (0.02)	–1 (0.02)	+1 (0.02)	5.84
34	+1 (2.0)	–1 (0.06)	–1 (0.06)	–1 (0.02)	–1 (0.02)	+1 (0.02)	3.53
35	–1 (1.0)	+1 (0.12)	–1 (0.06)	–1 (0.02)	–1 (0.02)	+1 (0.02)	6.16
36	+1 (2.0)	+1 (0.12)	–1 (0.06)	–1 (0.02)	–1 (0.02)	+1 (0.02)	4.83
37	–1 (1.0)	–1 (0.06)	+1 (0.12)	–1 (0.02)	–1 (0.02)	+1 (0.02)	4.72
38	+1 (2.0)	–1 (0.06)	+1 (0.12)	–1 (0.02)	–1 (0.02)	+1 (0.02)	3.77
39	–1 (1.0)	+1 (0.12)	+1 (0.12)	–1 (0.02)	–1 (0.02)	+1 (0.02)	6.29
40	+1 (2.0)	+1 (0.12)	+1 (0.12)	–1 (0.02)	–1 (0.02)	+1 (0.02)	2.33
41	–1 (1.0)	–1 (0.06)	–1 (0.06)	+1 (0.04)	–1 (0.02)	+1 (0.02)	7.11
42	+1 (2.0)	–1 (0.06)	–1 (0.06)	+1 (0.04)	–1 (0.02)	+1 (0.02)	1.61
43	–1 (1.0)	+1 (0.12)	–1 (0.06)	+1 (0.04)	–1 (0.02)	+1 (0.02)	3.52
44	+1 (2.0)	+1 (0.12)	–1 (0.06)	+1 (0.04)	–1 (0.02)	+1 (0.02)	5.87
45	–1 (1.0)	–1 (0.06)	+1 (0.12)	+1 (0.04)	+1 (0.04)	+1 (0.02)	5.91
46	+1 (2.0)	–1 (0.06)	+1 (0.12)	+1 (0.04)	+1 (0.04)	+1 (0.02)	4.42
47	–1 (1.0)	+1 (0.12)	+1 (0.12)	+1 (0.04)	+1 (0.04)	+1 (0.02)	5.03
48	+1 (2.0)	+1 (0.12)	+1 (0.12)	+1 (0.04)	+1 (0.04)	+1 (0.02)	5.77

Table 3 continued

Run	Flaxseed oil (%)	$NaNO_3$ (%)	K_2HPO_4 (%)	$MgSO_4$ (%)	KCl (%)	$FeSO_4 \cdot 7H_2O$ (%)	Activity U/mL
49	–1 (1.0)	–1 (0.06)	–1 (0.06)	–1 (0.02)	+1 (0.04)	+1 (0.02)	4.20
50	+1 (2.0)	–1 (0.06)	–1 (0.06)	–1 (0.02)	+1 (0.04)	+1 (0.02)	6.78
51	–1 (1.0)	+1 (0.12)	–1 (0.06)	–1 (0.02)	+1 (0.04)	+1 (0.02)	8.17
52	+1 (2.0)	+1 (0.12)	–1 (0.06)	–1 (0.02)	+1 (0.04)	+1 (0.02)	6.55
53	–1 (1.0)	–1 (0.06)	+1 (0.12)	–1 (0.02)	+1 (0.04)	+1 (0.02)	7.60
54	+1 (2.0)	–1 (0.06)	+1 (0.12)	–1 (0.02)	+1 (0.04)	+1 (0.02)	3.52
55	–1 (1.0)	+1 (0.12)	+1 (0.12)	–1 (0.02)	+1 (0.04)	+1 (0.02)	2.25
56	+1 (2.0)	+1 (0.12)	+1 (0.12)	–1 (0.02)	+1 (0.04)	+1 (0.02)	2.33
57	–1 (1.0)	–1 (0.06)	–1 (0.06)	+1 (0.04)	+1 (0.04)	+1 (0.02)	2.85
58	+1 (2.0)	–1 (0.06)	–1 (0.06)	+1 (0.04)	+1 (0.04)	+1 (0.02)	4.01
59	–1 (1.0)	+1 (0.12)	–1 (0.06)	+1 (0.04)	+1 (0.04)	+1 (0.02)	3.09
60	+1 (2.0)	+1 (0.12)	–1 (0.06)	+1 (0.04)	+1 (0.04)	+1 (0.02)	4.87
61	–1 (1.0)	–1 (0.06)	+1 (0.12)	+1 (0.04)	+1 (0.04)	+1 (0.02)	3.30
62	+1 (2.0)	–1 (0.06)	+1 (0.12)	+1 (0.04)	+1 (0.04)	+1 (0.02)	2.46
63	–1 (1.0)	+1 (0.12)	+1 (0.12)	+1 (0.04)	+1 (0.04)	+1 (0.02)	2.58
64	+1 (2.0)	+1 (0.12)	+1 (0.12)	+1 (0.04)	+1 (0.04)	+1 (0.02)	1.97
65	+α (0.09)	0 (0.09)	0 (0.09)	0 (0.03)	0 (0.03)	0 (0.015)	3.29
66	+α (2.91)	0 (0.09)	0 (0.09)	0 (0.03)	0 (0.03)	0 (0.015)	2.65
67	0 (1.5)	–α (0.005)	0 (0.09)	0 (0.03)	0 (0.03)	0 (0.015)	2.65
68	0 (1.5)	+α (0.175)	0 (0.09)	0 (0.03)	0 (0.03)	0 (0.015)	1.01
69	0 (1.5)	0 (0.09)	–α (0.005)	0 (0.03)	0 (0.03)	0 (0.015)	0.94
70	0 (1.5)	0 (0.09)	+α (0.175)	0 (0.03)	0 (0.03)	0 (0.015)	0.50
71	0 (1.5)	0 (0.09)	0 (0.09)	–α (0.002)	0 (0.03)	0 (0.015)	5.92
72	0 (1.5)	0 (0.09)	0 (0.09)	+α (0.058)	0 (0.03)	0 (0.015)	0.92
73	0 (1.5)	0 (0.09)	0 (0.09)	0 (0.03)	–α (0.002)	0 (0.015)	6.38
74	0 (1.5)	0 (0.09)	0 (0.09)	0 (0.03)	+α (0.058)	0 (0.015)	1.51
75	0 (1.5)	0 (0.09)	0 (0.09)	0 (0.03)	0 (0.03)	–α (0.001)	1.98
76	0 (1.5)	0 (0.09)	0 (0.09)	0 (0.03)	0 (0.03)	α (0.029)	1.33
77	0 (1.5)	0 (0.09)	0 (0.09)	0 (0.03)	0 (0.0)	0 (0.015)	10.02
78	0 (1.5)	0 (0.09)	0 (0.09)	0 (0.03)	0 (0.03)	0 (0.015)	9.72
79	0 (1.5)	0 (0.09)	0 (0.09)	0 (0.03)	0 (0.03)	0 (0.015)	9.06
80	0 (1.5)	0 (0.09)	0 (0.09)	0 (0.03)	0 (0.03)	0 (0.015)	9.38
81	0 (1.5)	0 (0.09)	0 (0.09)	0 (0.03)	0 (0.03)	0 (0.015)	9.85

Table 4 ANOVA analysis for the medium composition

	Sum of squares	Degrees of freedom	Mean squares	*F* test
Regression	916.1	24	38.2	8.7
Lack of fit	219.5	52	4.2	
Pure error	0.6	4	0.2	
Total	1136.1	80		

$F_{24;\ 56;\ 0.05} = 1.70$; $R^2 = 0.80$ and p value < 0.05

(ANOVA) described in Table 6, shows that 81% of the results regarding the production of cutinase after 48 h of fermentation were explained by regression and 19% by the residues. There was a lack of fit to the model, indicated by the comparison between $F_{calc} = 2.58$ and $F_{5;\ 7;\ 0.05} = 3.97$. This fact was confirmed after analysis of the contour curve and response surface, displayed in Fig. 2.

Effect of the inoculum

The effect of the inoculum cell concentration on the production of cutinase was studied, employing the Tukey test at a confidence interval of 95% and a significance level of $p \leq 0.05$, and the software "Statistica for Windows" (Microsoft, version 5.0, 1995) for data analysis. The tests were done in duplicate.

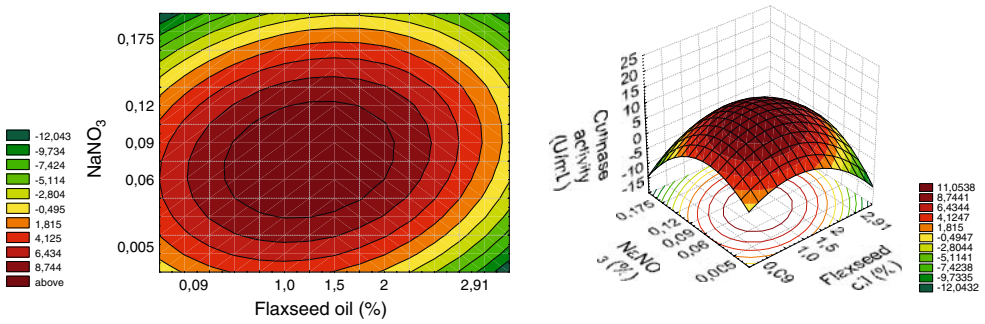

Fig. 1 Response surface and contour diagram for cutinase activity considering flaxseed oil and $NaNO_3$

Table 5 Optimization of fermentation conditions for cutinase production (U/mL), according to a central composite design (CCD), analysed after 48 h of fermentation

Run	Rotational speed	Temperature °C	Cutinase activity U/mL
1	–1 (115)	–1 (26)	6.25
2	+1 (185)	–1 (26)	10.40
3	–1 (115)	+1 (34)	4.62
4	+1 (185)	+1 (34)	0.86
5	–α (100)	0 (30)	16.44
6	+α (200)	0 (30)	5.96
7	0 (150)	–α (25)	1.53
8	0 (150)	+α (35)	0.85
9	0 (150)	0 (30)	7.70
10	0 (150)	0 (30)	8.61
11	0 (150)	0 (30)	8.60
12	0 (150)	0 (30)	9.57
13	0 (150)	0 (30)	9.48

Table 6 ANOVA analysis for the fermentation conditions

	Sum of squares	Degrees of freedom	Mean squares	*F* test
Rotation (*L*)	26.0	1	26.0	44.48
Rotation (*Q*)	6.6	1	6.6	11.32
Temperature (*L*)	22.9	1	22.9	39.19
Temperature (*Q*)	99.4	1	99.4	169.75
Rotation *L* × Temperature *L*	15.6	1	15.6	26.73
Regression	179.2	5	35.8	2.58
Lack of fit	40.2	3	13.3	22.93
Pure error	2.3	4	0.6	
Total	221.8	12		

$F_{5;\ 7;\ 0.05} = 3.97$; $R^2 = 0.76386$ and p value < 0.05

The cutinase activity was 22.68 ± 0.79, 18.40 ± 0.08 and 5.98 ± 0.44 U/mL with the use of an inoculum containing 12.72×10^7, 6.2×10^7 and 3.75×10^7 spores/mL, respectively.

Using this approach, the authors demonstrated a significant difference between the three dilutions in the production of cutinase after 48 h. It was not possible to increase the inoculum concentration further due to limitations of the method. The value of 12.72×10^7 spores/mL was then used as the standard for the inoculum.

The importance of the inoculum size and consistency has long been recognized to determine the productivity of industrial fermentations. In 1993, Webb and Kamat [22] using *Saccharomyces cerevisiae*, demonstrated that the process of inoculation by liquid transfer has the lowest level of variability, which was considered well suited for use in industrial processes.

Conclusions

The search for cutinase inducers other than cutin, which is expensive and difficult to obtain in large scale, is an important issue affecting the viability of production of fungal cutinases by industrial fermentation.

Between the substances studied, flaxseed oil showed the best activity as a cutinase inducer, with a yield of 15.48 U/mL in the univariate analysis, versus 11.34 U/mL with cutin.

Between the nitrogen sources, the best result was obtained with yeast extract. However, the cutinolytic activity obtained was less than that of cutin.

In the optimization of the growth medium using experimental planning, the best results obtained for cutinolytic activity were 18.84 U/mL, using 1.0% flaxseed oil, 0.06% $NaNO_3$, 0.06% K_2HPO_4, 0.02% $MgSO_4$, 0.02% KCl and 0.01% $FeSO_4 \cdot 7H_2O$. The optimization of the culture medium is a relevant aspect in the development of fermentation processes. In the experimental validation of the optimized composition of the growth medium, the authors obtained a mean cutinolytic activity of 17.52 U/mL with a standard deviation of ±6%. The authors considered these results as confirmation of the CCD results, thus establishing the optimized conditions for the production of cutinase by *F. oxysporum.*

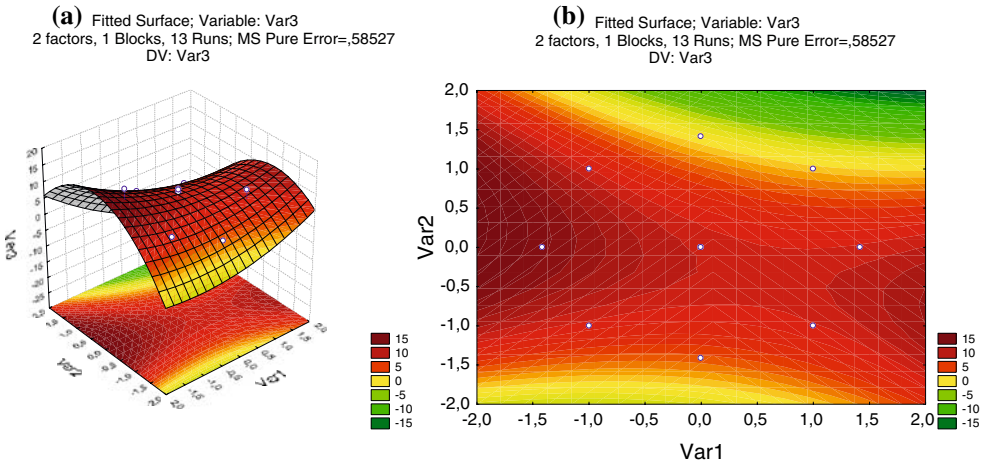

Fig. 2 Contour curve (**a**) and response surface (**b**) describing the effect of agitation (rpm) as a function of temperature (°C)

The analysis of the fermentation conditions using the CCD strategy registered the best production of cutinase with the use of 30 °C and 100 rpm.

With respect to the inoculum, the best cutinase production was 22.68 U/mL using an aliquot of 12.72×10^7 spores/mL.

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References

1. Borreguero I, Carvalho CML, Cabral JMS, Sinisterra JV, Alcântara AR (2001) Enantioselective properties of *Fusarium solani pisi* cutinase on transesterification of acyclic diols: activity and stability evaluation. J Mol Catal B Enzym 11:613–622
2. Calado CRC, Ferreira BS, Fonseca MMR, Cabral JMS, Fonseca LP (2004) Integration of the production and the purification process of cutinase secreted by a recombinant *Saccharomyces cerevisiae* SU 50 strain. J Biotechnol 109:147–158
3. Carvalho CML, Serralheiro MLM, Cabral JMS, Aires-Barros MR (1997) Application of factorial design to the study of transesterification reactions using cutinase in AOT-reversed micelles. Enzyme Microb Technol 22:117–123
4. Christakopoulos P, Mamma D, Nerinckx W, Kekos D, Macris B, Claeyssens M (1996) Production and partial characterization of xylanase from *Fusarium oxysporum*. Bioresour Technol 58:115–119
5. Christakopoulos P, Tzalas B, Mamma D, Stamatis H, Liadakis GN, Tzia C, Kekos D, Kolisis FN, Macris BJ (1998) Production of an esterase from *Fusarium Oxysporum* catalysing transesterification reactions in organic solvents. Process Biochem 33:729–733
6. Egmond MR, De Vlieg J (2000) *Fusarium solani pisi* cutinase. Biochem 82:1015–1021
7. Fett WF, Gerard HC, Moreau RA, Osman SF, Jones LE (1992) Cutinase production by *Streptomyces* spp. Curr Microb 25:165–171
8. Fett WF, Wijey C, Moreau RA, Osman SF (1999) Production of cutinase by *Thermomonospora fusca* ATCC 27730. J Appl Microb 86:561–568
9. Gonçalves AMD, Aires-Barros MR, Cabral JMS (2003) Interaction of an anionic surfactant with a recombinant cutinase from *Fusarium Solani pisi*: a spectroscopic study. Enzyme Microbial Technol 32:868–879
10. Hajjar AB, Nicks PF, Knowles J (1990) Preparation of monomeric acrylic ester intermediates using lipase catalysed transesterifications in organic solvents. Biotechnol Lett 12:825–830
11. Hedström G, Backlund M, Slotte JP (1993) Enantioselective synthesis of ibuprofen esters in AOT/isooctane microemulsions by *Candida cylindracea* lipase. Biotechnol Bioeng 42:618–624
12. http://www.brenda.uni-koeln.de, accessed in 08/15/2006
13. Kerry NL, Abbey M (1997) Red wine and fractionated phenolic compounds prepared from red wine inhibit low density lipoprotein oxidation in vitro. Ather Limerick 135:93–102
14. Liu J, Xing J, Chang T, Ma Z, Huizhou L (2005) Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Process Biochem 40:2757–2762
15. Loomer S, Adlercreutz P, Mattiasson B (1990) Triglyceride interesterification by lipases. 1. Cocoa butter equivalents from a fraction of palm oil. J Am Oil Chem Soc 67:519–524
16. Macedo GA, Pio TF (2005) A rapid screening method for cutinase producing microorganisms. Braz J Microb 36:388–394
17. Melo EP, Baptista RP, Cabral JMS (2003) Improving cutinase stability in aqueous solution and in reverse micelles by media engineering. J Mol Catal B Enzym 22:299–306
18. Rispoli FJ, Shah V (2007) Mixture design as a first step for optimization of fermentationmedium for cutinase production from *Colletotrichum lindemuthianum*. J Ind Microb Biotechnol 34:349–355
19. Serralheiro MLM, Prazeres DMF, Cabral JMS (1994) Dipeptide synthesis and separation in a reverse micellar membrane reactor. Enzyme Microb Technol 16:1064–1073
20. Sreenivasan B (1978) Interesterification of fats. J Am Oil Chem Soc 55:796–805
21. Walton TJ, Kollattukuy PE (1972) Determination of the structures from cutin monomers by a novel depolymerization procedure and combined gas chromatography and mass spectrometry. Biochemistry 11:1885–1896
22. Webb C, Kamat SP (1993) Improving fermentation consistency through better inoculum preparation. World J Microb Biotechnol 9:308–312