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Production of Pectinases by *A. niger*: Influence of Fermentation Conditions

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ABSTRACT

Response surface methodology was used for optimization of polygalacturonase (PG) and pectinesterase (PE) production in submerged fermentation by A.niger. A Central Composite Experimental Design was applied, consisting of 22 experiments, including eight central points. Variables studied were: fermentation time (24 to 120 h), pH (3.5 to 6.5) and initial concentration of pectin (5 to 20 g/l). Maximum PE production was 220 U/l, after 74 h of culture, in a medium containing 20 g/l of pectin (pH 6.5). The optimal conditions for PG production were pH: 4.1, 20 g/l of pectin and 94 h of fermentation with a maximum value of 1032 U/l. Under these conditions, the PE production was low (15 U/l). A liquid extract with high PG activity and low PE activity could be suitable to be used in food processing in order to reduce the production of methanol.

Key words*: Pectinases; Polygalacturonase; Pectinesterase; Aspergillus niger;* Response surface methodology; Citrus pectin

INTRODUCTION

Pectinases are widely used in industrial processing of fruits and vegetables, because they reduce the viscosity of juices and facilitate extraction, maceration, liquefaction and clarification processes (Naidu and Panda, 1999).

Polygalacturonase (PG) is a depolymerizing enzyme that cleaves glycosidic bonds of pectins by means of hydrolysis. Pectinesterase (PE) is the pectolytic enzyme that catalyzes the hydrolysis of ester links of the pectin molecule. Action of both enzymes is needed in order to obtain complete hydrolysis of the pectin molecule (Taragano and Pilosof, 1999).

Aspergillus niger strains are widely used in several fermentation processes for the production of pectic enzymes. The synthesis of pectinases is induced by pectin or for some of its derivatives (Solís Pereira et al., 1993). The cell growth, sporulation and production of the enzymes can be affected by the composition of the medium and fermentation conditions (Maldonado and Callieri, 1989; Costa et al., 2007).

Most reported studies are carried out varying one factor at a time while keeping the others at a constant level. This approach does not depict the effects of the interactions between the variables.

In the present work, response surface methodology was applied to optimize pH, initial pectin concentration and fermentation time conditions on

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the production of PG and PE by *A. niger* in submerged fermentation.

MATERIALS AND METHODS

Fermentation medium

The liquid medium used contained (g/l): KH_2PO_4, 4; Na_2HPO_4, 6; $FeSO_4$ $7H_2O$, 0.01; $CaCl_2$, 0.01; $MgSO_4$ $7H_2O$, 0.2; $(NH_4)_2$ SO_4, 2; H_3BO_3, 10 μg/l; $MnSO_4$, 10 μg/l; $ZnSO_4$ $7H_2O$, 70 μg/l; citrus pectin (Parafarm), 5-20 g/l (Maldonado et al., 1996).

Pectin agar: Idem fermentation medium, citrus pectin 15 g/l; agar 15 g/l.

Microorganism

A. niger Nº 300, isolated in our laboratory from mouldy citrus fruit peels, was maintained in pectin agar slants. The inoculum was prepared by stirring one-week-old slants with a sterile solution of 0.05% Tween 80 in water to obtain a 1.25 10^7 spores/cm^3 suspension.

Enzyme production

Two hundred and fifty millilitres Erlenmeyers flasks with 45 ml of medium were inoculated with 5 ml of the inoculum and then incubated at 30 ºC at different times on a rotary shaker at 200 rpm.

The biomass was separated by filtration through a paper filter (Whatman Nº1). The filtrate was stored at –18 ºC until assayed.

Enzyme assays

PG activity was determined by incubating 4 ml of 0.5% polygalacturonic acid (Sigma) in 0.2 M acetate buffer (pH 4.5) with 1 ml of the enzyme extract. Reaction was carried out at 37 ºC for 60 min. The release of reducing groups was determined by the dinitrosalicylic acid method (Miller, 1959). A calibration curve was made using galacturonic acid (Sigma) as standard. One unit of PG was defined as the amount of enzyme that released 1 μmol of galacturonic acid per minute (Maldonado and Strasser de Saad, 1998).

PE activity was measured by adding 2 ml of the enzyme extract to 10 ml of 0.5 % citrus pectin (Sigma) in 0.1 M NaCl, the pH was adjusted to 4.5 with 0.1 M NaOH. The reaction was carried out at 35 ºC for 60 min. The amount of equivalents due to the carboxyl groups released was measured by titration with 0.02 N NaOH. One unit of PE was defined as the amount of enzyme that released 1 μmol of carboxyl groups per minute (Maldonado et al., 1996).

Experimental design

The experimental design selected analyzed three variables at five levels (design type CCD = 22 runs, including eight central points for experimental error detection) (Freund and Wilson, 1997). Variables studied were: fermentation time (24 to 120 h), initial pH (3.5 to 6.5) and initial pectin concentration (5 to 20 g/l).

Real and transformed variables according to the experimental design selected are presented in Table 1.

Regression linear analysis was used to eliminate terms of $P > 0,05$.

All the experiments were conducted in triplicate and the results showed the mean values of the activities.

Table 1 - Experimental variables in real and transformed values

	Time (h)	PH	Pectin (g/l)
+α*	120	6.5	20.0
+1	100.54	5.892	16.956
0	72	5.0	12.5
-1	43.46	4.108	8.040
-α*	24	3.5	5.0

* α = 1.68179.

RESULTS AND DISCUSSION

Table 2 presents the treatment combinations and responses. The regression coefficients of the equations relating the enzymes production with the variables studied and the respective levels of significance are shown in Table 3.

Figs 1 to 4 show the response surfaces and contour plots for PG and PE production as a function of two independent variables with the other one at a

given constant level.

All the equations presented a very good adjustment with experimental data (R^2 coefficients > 0.8) (Table 3).

The three independent variables had significant linear effects on PG production (Table 3). Pectin had a linear and positive effect: a high PG production was observed when pectin concentration was increased (Fig. 1).

The negative values of quadratic effects for pH and time indicated the existence of a maximum as a function of these variables (Table 3).

Optimum PG production was observed for pH 4.1 and 94 h of culture (Fig. 2).

Table 2 - Treatment combinations and mean responses.

Run	Pectin (g/l)	Time (h)	pH	PG (U/l)	PE (U/l)
1	-1	-1	-1	654	33
2	0	-α	0	726	23
3	-1	+1	-1	915	51
4	-1	-1	+1	650	57
5	0	0	0	799	58
6	0	0	0	863	49
7	0	0	0	881	51
8	0	+α	0	852	33
9	0	0	0	958	60
10	+1	+1	+1	829	142
11	0	0	+α	608	210
12	+1	-1	+1	676	72
13	0	0	0	872	59
14	+α	0	0	988	50
15	0	0	0	808	57
16	-α	0	0	680	7
17	-1	+1	+1	685	33
18	0	0	0	898	57
19	+1	+1	-1	923	42
20	+1	-1	-1	862	37
21	0	0	-α	934	69
22	0	0	0	898	43

PG: polygalacturonase activity. PE: pectinesterase activity.

Table 3 - Regressions coefficients and levels of significance.

	PG activity		PE activity	
	Constant	**Level of significance**	**Constant**	**Level of significance**
Independent term	-461.541		857.421	
Time	7.65078*	0.0035	1.92157*	
pH	405.149*	0.0001	-378.546*	0.0001
Pectin	14.8432*	0.0006	-3.68687*	0.0153
pH^2	-49.2359*	0.0158	35.8914*	0.0000
t^2	-0.0402687*	0.0380	-0.0133442*	0.0390
$Pectin^2$	**		-0.537691*	0.0419
pH * pectin	**		4.0541*	0.0292
R^2	0.81		0.86	

* Significant (P < 0,05), ** not significant (P> 0,05)

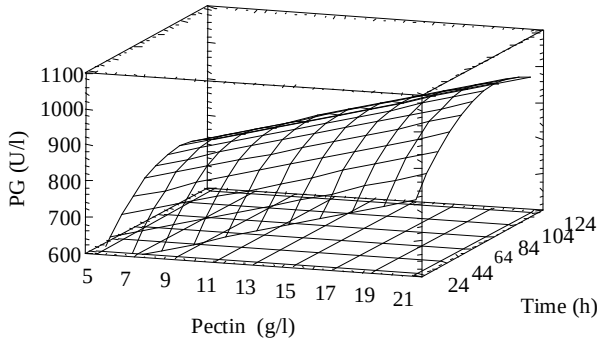

Figure 1 - Effects of fermentation time and initial pectin on PG production (pH: 5.0).

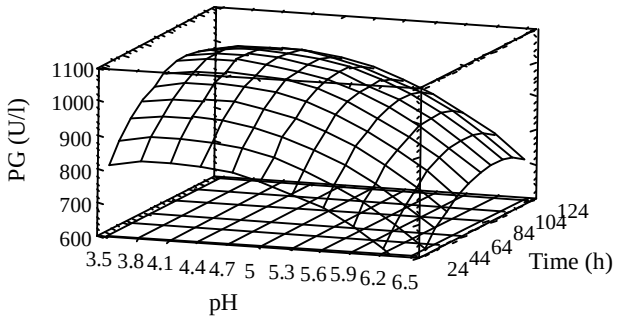

Figure 2 - Effects of fermentation time and initial pH on PG production (initial pectin concentration: 20 g/l).

No significant interactions were observed among the variables (Table 3).

Low PG values obtained at about pH 6.5 were in agreement with those reported by Solís Pereira et al. (1993). Acuña-Argüelles et al. (1995) studied the stability of *Aspergillus niger* CH4 PG in submerged fermentation as a function of pH and observed that the enzyme was quickly denatured for pH values higher than 5.0.

Low initial pectin concentrations yield low pectinases production, since pectinases are generally inducible enzymes (Naidu and Panda, 1998; Malvessi and Silveira, 2004).

The three variables studied had significant linear effects on PE production (Table 3).

The pH showed positive quadratic effects, indicating a minimum as a function of this variable at the range 4.2 - 4.8 (Fig. 3).

Interaction between pH and pectin was significant and positive (Table 3), and low pH values did not affect PE production, but the effect was strongly positive at higher pH values (Fig. 3). The highest PE production was obtained at pH 6.5 and 20 g/l of pectin.

Table 4 showed that PE production slightly increased at about 74 h of fermentation.

At pH higher than 5.0, PE production increased with pectin since the synthesis of this enzyme was induced by this substrate(Maldonado and Callieri, 1989; Maldonado et al., 1994).

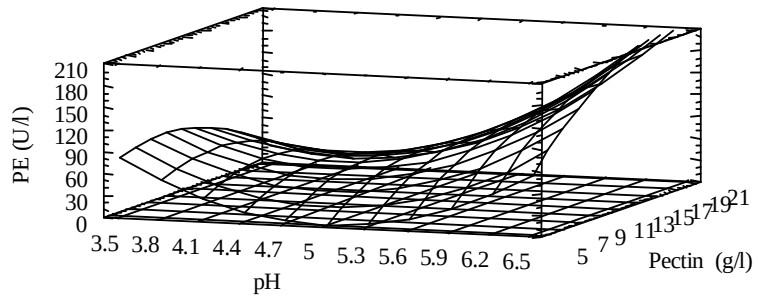

Figure 3 - Effects of initial pectin concentration and pH on PE production (fermentation time: 72 h).

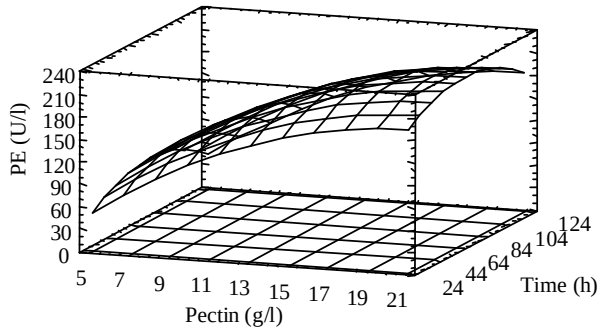

Figure 4 - Effects of fermentation time and initial pectin concentration on PE production (pH: 6.5).

CONCLUSIONS

Maximum PE production was 220 U/l at 74 h of culture, 20 g/l of pectin and pH 6.5.

Optimum conditions for PG production were 94 h of incubation, 20 g/l of pectin and pH 4.1. Under these conditions PG production was 1032 U/l and PE production was only 15 U/l.

A liquid extract with a high PG activity would accelerate the depolymerization of the pectin molecule while a low PE activity could be advantageous for processing juices taking into account PE liberated methanol that would damage the volatile ester content responsible for the specific scent of fruits.

RESUMO

A metodologia de superfície de resposta foi utilizada para a otimização da produção de poligalacturonasa (PG) e pectinesterasa (PE), por A. niger em fermentação submergida. Foi aplicado um Desenho Experimental Composto Central abrangendo 22 experiências, incluindo oito pontos centrais. As variáveis estudadas foram: tempo de fermentação (24 a 120 h), pH (3.5 a 6.5) e concentração inicial de pectina (5 a 20 g/l). A produção máxima de PE foi de 220 U/l, após 74h de cultivo, 20 g/l de pectina e pH 6.5. As condições ótimas para a produção de PG foram pH 4.1, 20 g/l de pectina e 94 h de fermentação, com um valor máximo de 1032 U/l. Sob estas condições, a produção de PE foi baixa (15 U/l). Um extrato líquido com alta atividade PG e baixa atividade PE poderia ser conveniente para ser utilizado no processamento e alimentos, visando reduzir a produção de metanol.

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