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Influence of carbon source, pH, and temperature on the polygalacturonase activity of *Kluyveromyces marxianus* CCMB 322

Influência da fonte de carbono, pH e temperatura na atividade da enzima poligalacturonase de Kluyveromyces marxianus CCMB 322

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Abstract

Microbial pectinolytic enzymes are known to play a commercially important role in a number of industrial processes. Two kinds of yeast can be discerned regarding the production of enzymes. One group includes those which can produce enzymes in the absence of an inducer, and the other group comprises the yeasts that produce enzymes in the presence of an inducer. The objective of this study was to investigate the influence of pectic substances, glucose, pH, and temperature on the polygalacturonase activity by *Kluyveromyces marxianus* CCMB 322. The yeast was grown in a fermentation broth containing different concentrations of glucose and pectic substances. The polygalacturonase activity was determined by the DNS method, and the pH and temperature were optimized using a central composite experimental design. The polygalacturonase secreted by *K. marxianus* CCMB 322 was partially constitutive showing optimum pH and temperature of 7.36 and 70 °C, respectively, and maintained approximately 93% of its original activity for 50 minutes at 50 °C. Thermal stability of the polygalacturonase enzyme was studied at different temperatures (50, 60, 70, and 80 °C) and different incubation times (0, 10, 20, 30, 40, and 50 minutes). This study showed that glucose can influence the regulation of the synthesis of polygalacturonase.

Keywords: yeasts; enzymes; thermostability; pectic substances; biotechnology.

Resumo

Enzimas pectinolíticas de origem microbiana são conhecidas por desempenhar um papel importante comercialmente em uma série de processos industriais. Dois tipos de levedura podem ser distinguidos para a produção dessas enzimas. Um grupo inclui aqueles que têm capacidade de produzi-las na ausência de um indutor e outro grupo compreende as leveduras que as produzem na presença de um indutor. O objetivo deste estudo foi investigar a influência de substâncias pécicas, de glicose, do pH e da temperatura na atividade da poligalacturonase de *Kluyveromyces marxianus* CCMB 322. O cultivo foi em caldo de fermentação contendo diferentes concentrações de glicose e substâncias pécicas. A atividade da poligalacturonase foi determinada pelo método do DNS, e pH e temperatura otimizados pelo delineamento experimental central composto de experimentos. A poligalacturonase secretada por *K. marxianus* CCMB 322 foi parcialmente constitutiva, apresentando pH e temperatura ótima de 7,36 e 70 °C, respectivamente e reteve 93% de sua atividade original após 50 minutos a 50 °C. A termoestabilidade da enzima poligalacturonase foi estudada em várias temperaturas (50, 60, 70 e 80 °C) em diferentes tempos de incubação (0, 10, 20, 30, 40 e 50 minutos). Este estudo mostrou que a glicose tem influência na regulação da síntese da poligalacturonase.

Palavras-chave: leveduras; enzimas; termoestabilidade; substâncias pécicas; biotecnologia.

1 Introduction

In recent years, the potential of using microorganisms as a biotechnological source of industrially relevant food processing enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity. Enzymes which hydrolyze pectic substances are broadly known as pectinases that include polygalacturonase, pectin esterase, pectin lyase, and pectate

lyase on the basis of their mode of action (SCHWAN; ROSE, 1994).

Chemically, pectic substances are complex colloidal acid polysaccharides with a backbone of galacturonic acid residues linked by $\alpha(1-4)$ linkages (KASHYAP et al., 2001). Pectinases are mainly used for increasing filtration efficiency and clarification

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of fruit juices, as well as in maceration, liquefaction, and extraction of vegetable tissues. Various literature studies and reviews on the production and industrial applications of pectinases are available (ALKORTA et al., 1997; BLANCO; SIEIRO; VILLA, 1999; HOONDAL et al., 2002; JAYANI; SAXENA; GUPTA, 2005).

Pectic enzymes produced by microorganisms origin account for 25% of all enzymes used in the food industry and have been traditionally used in the extraction and clarification of fruit juice (JAYANI; SAXENA; GUPTA, 2005). In addition, the use of high temperatures during the processing of juice products is an important step in industrial processes (GOMES et al., 2007). Thus, enzyme thermal stability has advantages for application in industry (JAENICKE, 1999).

Polygalacturonases (EC 3.2.1.15) hydrolyze the polygalacturonic acid chain by addition of water and are the most abundant among all of the pectinolytic enzymes (GUMMADI; PANDA, 2003; JAYANI; SAXENA; GUPTA, 2005).

Recent surveys have underlined the potential of natural environments as sources for the isolation and selection of microorganisms to be used as biotechnological sources of industrially relevant enzymes (BUZZINI; MARTINI, 2002). The Brazilian semi-arid zone is located almost exclusively in the Northeast of the country (<http://www.uefs.br/ppbio/home.htm>).

The semi-arid is a great field for biotechnology exploration because yeasts adapted to the significantly heterogeneous physical environment, including different climates, system rains, relief, soil, and hydrography may be endowed with its own characteristics with peculiarities of industrial interest (OLIVEIRA et al., 2009).

According to Galvagno and Forchiassin (2004), there are enzymes which are necessary for the basic metabolism of a fungus and are known as constituent enzymes. On the other hand, the enzymes that require the presence of the substrate to induce their synthesis and activity are known as inducible enzymes. Moreover, according to these authors, fungi can produce adaptive enzymes in the presence of a specific substrate.

Said and Pietro (2002) consider that the production of extracellular pectinases by microorganisms often requires an inducer, for instance, citrus pectin. In some situations, the major carbon source can also act as inducer. In addition, these enzymes are influenced by pH and cultivation temperature (McKAY, 1988).

The aim of this study was to analyze the influence of pectic substances, glucose, pH, and temperature (°C) on the polygalacturonase activity by *Kluyveromyces marxianus* CCMB 322.

2 Materials and methods

2.1 Microorganism

The yeast strain *Kluyveromyces marxianus* CCMB 322 used in this study was isolated from necrotic plant tissue (*Agave* sp.) collected from natural environments of Brazilian

semi-arid region, Bahia, Brazil and obtained from the Culture Collection of Microorganisms of Bahia (CCMB) from UEFS, Brazil. Molecular identification was performed according with Oliveira et al. (2009).

2.2 Polygalacturonase production

The yeast strain of isolate *Kluyveromyces marxianus* CCMB 322 was previously grown on YM agar (Difco, USA) at 28 °C for 18 hours, diluted in sterile distilled water to about 10^8 cells.mL⁻¹ and 10% (v/v), and inoculated in 25 mL flasks containing mineral medium for fermentation (PATCHING; ROSE, 1969), which contained the following (g.L⁻¹): 3 (NH₄)₂SO₄, 4.5 KH₂PO₄, 0.25 MgSO₄, and 0.25 CaCl₂, pH 5.0 and was supplemented with different combinations of 10 g.L⁻¹ glucose, citrus pectin (Sigma), galacturonic acid (Sigma), polygalacturonic acid (Sigma), and esterified pectin (Sigma). After incubation at 28 °C for 48 hours on an orbital shaker at 150 rev/minutes, the microorganisms were separated by centrifugation at $10.000 \times g$ for 10 minutes at 4 °C. The culture media supernatants were used as extracellular fractions.

The cells separated by centrifugation were used were dried to constant weight at 85 °C.

2.3 Enzyme assay

Polygalacturonase activity was assayed spectrophotometrically (A_{540}) using the dinitrosalicylic reagent (Sigma Chemical Co. (St Louis, MO, USA) method described by Miller (1959). The reaction mixture consisted of 250 µL of 1% (w/v) polygalacturonic acid (Sigma Chemical Co., St Louis, MO, USA) in 50 mM acetate buffer, pH 4.5, and 350 µL of culture supernatant. The reaction mixture was incubated for 60 minutes at 45 °C. After incubation, the mixture was boiled at 100 °C for 15 minutes. After cooling, 6 mL of distilled water were added to the reaction medium. The galacturonic acid was measured by reaction with dinitrosalicylic reagent at 540 nm (UV-Visible spectrophotometer - Varian-Cary 50). One unit of enzyme activity (UA) of polygalacturonase was defined as the amount of the enzyme that catalyzed the formation of 1 µmol of galacturonic acid min⁻¹.

2.4 Experimental design for determination of optimum pH and temperature

A central composite design (CCD) for two factors and three levels leading to 11 sets of experiments (assays), performed in triplicate, was used to determine the optimum pH and temperature. The response surface analysis was based on multiple linear regressions taking into account the main quadratic and interaction effects according to the following Equation 1:

$$Y = \beta_0 + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where Y is the predicted response; β_0 , the constant; β_1 and β_2 , linear effect; β_{11} and β_{22} , second order effect; and β_{12} , the interaction effect between variables 1 and 2.

2.5 Thermal stability of polygalacturonase enzyme

The culture supernatant obtained before, as explained in the section 2.2, was exposed to various incubation temperatures (50, 60, 70, and 80 °C) and different incubations times (0, 10, 20, 30, 40, and 50 minutes). The residual activities were determined as described in 2.4.

2.6 Statistical analysis

The data were analyzed using STATISTIC 6.0 software (StatSoft, Inc., OK) to generate a design matrix and a dimensional response surface plot, and for the analysis of variance (ANOVA) and Tukey test (5%).

3 Results and discussion

3.1 Influence of pectic substances and glucose

An increase in extracellular polygalacturonase activity (Table 1) when the yeast was grown in the combination of glucose (1%) and pectin substances (1%), as compared to the individual substrates, suggests that the extracellular polygalacturonase production of the *Kluyveromyces marxianus* CCMB 322 was partially constitutive, as described in a similar study by Winborne and Richard (1978).

The presence of galacturonic acid (1%) in the media containing glucose (1%) causes an increase in enzyme activity from 0.2126 to 0.7457 µmol/mL/minutes, which represents an increase of about 350% in the pectinolytic activity (Table 1). Winborne and Richard (1978) reported that the addition of pectin to the culture medium resulted in an increase of about 100% in the enzyme activity secreted by *Kluyveromyces fragilis* (*Kluyveromyces marxianus*).

Low levels of polygalacturonase, except for galacturonic acid, were produced in the absence of glucose suggesting that *Kluyveromyces marxianus* (CCMB 322) can use pectin

substances as energy source to promote cell growth and polygalacturonase production (Table 1).

These results differ from those of McKay (1988), who reported the ability of yeast strain *Kluyveromyces marxianus* NCYC 587 to produce polygalacturonase constitutively during growth on glucose, which will not grow with polygalacturonic acid as sole carbon and energy source. In addition, a study with a strain of *Kluyveromyces marxianus* CCT 3172, published by Schwan and Rose (1994), reported that no significant increase in growth or enzyme secretion was found when pectin, pectic acid, or polygalacturonic acid was added to media containing 1% (w/v) glucose.

According to Blanco, Sieiro and Villa (1999), the influence of other parameters such as the pH of the medium, inoculum size, incubation time, or the addition of nitrogen sources in the screening of pectic enzymes has received less attention.

According to Schmidell (2001), the regulatory study on the synthesis and excretion of enzymes by microorganisms is a cellular metabolism aspect of great interest to the enzyme industry and depends on the strain selected, the fermentation process, and the characteristics of the culture medium.

3.2 Determination of optimum pH and temperature

Eleven experiments were performed under different combinations of pH and temperature, and the activity measurements obtained are shown in Table 2. By applying multiple regression analysis on the experimental data, the following polynomial Equation 2 was found to explain the activity data:

$$Y = -0.176 + 0.0472X_1 - 0.00322X_1^2 + 0.00112X_2 - 0.00000816X_2^2 + 0.00000331X_1X_2 \quad (2)$$

where X_1 is the coded value for temperature (°C) and X_2 is the coded value for pH.

The response surface plot (Figure 1) indicates that the polygalacturonase secreted by *K. marxianus* CCMB 322 has optimum pH and temperature of 7.36 and 70 °C, respectively (during 48 hours of incubation).

The predicted relative specific activity was compared with the experimental values, which are given in Table 1. The coefficient of determination R^2 , calculated as 0.89, implies that 89% of total variation in the residual activities is explained by the fitted model. Statistical testing of the model was carried out by Fisher's statistical test for analysis of variance (Table 3). The chart of Pareto (Figure 2) shows that the variables pH and temperature are linearly and quadratically significant for the activity polygalacturonase of *Kluyveromyces marxianus* CCMB 322, because both variables have a P value greater than 0.05.

The results show that the higher activity of polygalacturonase from *Kluyveromyces marxianus* CCMB is obtained between pH 6.2 and 8.5, with the optimum pH at 7.36. Temperatures from 60 to 80 °C proved to be the optimum range to activity production, with the optimum value occurring at 70 °C

Table 1. Dry biomass and polygalacturonase activity produced by *Kluyveromyces marxianus* CCMB 322, grown in mineral medium for fermentation, supplemented with different combinations of glucose and pectic substance, at 28 °C for 48 hours.

Carbon source from the basal medium	Dry biomass (mg)	Polygalacturonase activity (µmol/mL/minutes)*
Glucose	260	0.2126 ^a
Glucose + citrus pectin	342	0.3035 ^b
Glucose + pectin esterified	308	0.3496 ^c
Glucose + polygalacturonic acid	601	0.2305 ^d
Glucose + galacturonic acid	207	0.7457 ^e
No glucose + citrus pectin	73	0.0769 ^f
No glucose + pectin esterified	65	0.0456 ^g
No glucose + polygalacturonic acid	469	0.0882 ^f
No glucose + galacturonic acid	19	0.4851 ^h

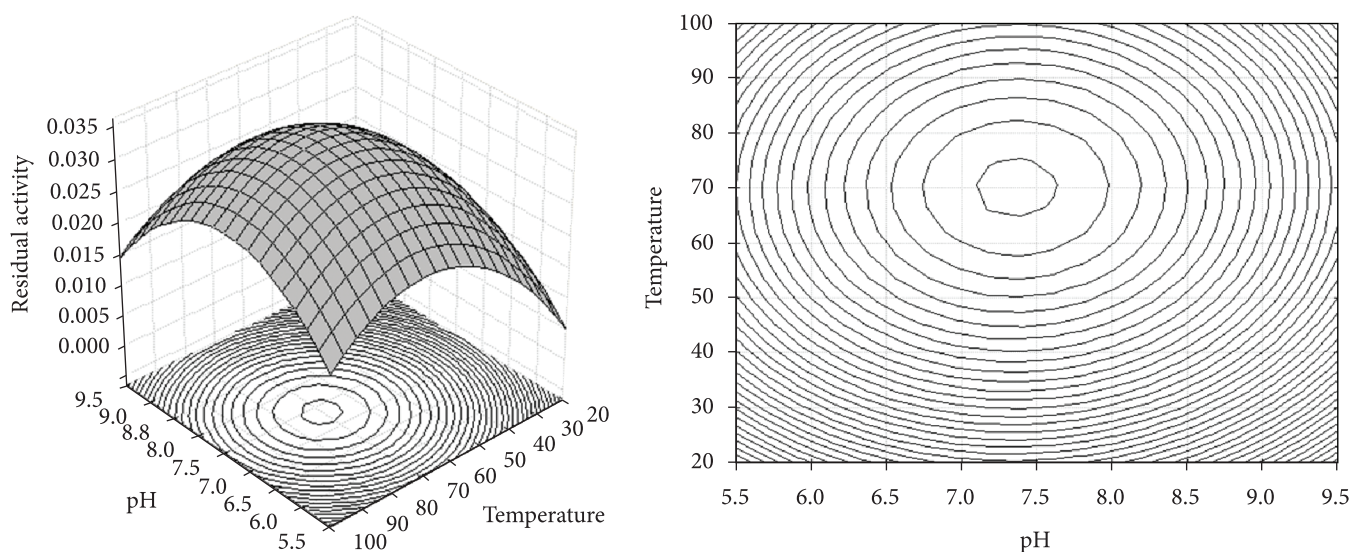
*Means of specific activity followed by the same uppercase superscript letter did not significantly differ by the Tukey test ($p < 0.05$).

Table 2. Experimental and predicted activities of polygalacturonase after 15 minutes of incubation.

Experiment(°)	X ₁ = pH	X ₂ = Temperature	Residual activity experimental (U)	Residual activity predicted (U)
1	6.4 (-1)	38.7 (-1)	0.0237	0.0254
2	6.4 (-1)	81.3 (+1)	0.0312	0.0322
3	8.6 (+1)	38.7 (-1)	0.0216	0.0231
4	8.6 (+1)	81.3 (+1)	0.0295	0.0303
5	6 (-1.4)	60 (0)	0.0309	0.0295
6	9 (+1.4)	60 (0)	0.0279	0.0267
7	7.5 (0)	30 (-1.4)	0.0248	0.0231
8	7.5 (0)	90 (+1.4)	0.0337	0.0329
9	7.5 (0)	60 (0)	0.0353	0.0353
10	7.5 (0)	60 (0)	0.0355	0.0353
11	7.5 (0)	60 (0)	0.0351	0.0353

Table 3. Analysis of variances in the regression model for optimization of pH and temperature of enzyme poligalacturonase of *Kluyveromyces marxianus*.

Source of variations	SQ (Sum Quadratic)	Degree of freedom	Mean square	F calculated	F tabulated (IC de 95%)	Determination coefficient (R ²)
Regression	0.000227	4	0.00006925	29.67	9.1	
Residual	0.000014	6	0.00000233			
Lack of fit	0.000000	1	0.000000	0.00863	18.51	0.89
Pure error	0.000014	5	0.000003			
SQ total	0.000241	10				

**Figure 1.** Response surface plot and the corresponding contour plot showing the effects of pH and temperature (°C) on the pectinolytic activity of *Kluyveromyces marxianus* CCMB 322 after 48 hours of incubation.

(Figure 1). The results of the factorial design generated by the matrix (Table 2) show that the effects of temperature and pH were significant.

The studies of Moyo et al. (2003) showed that polygalacturonase from *Kluyveromyces wickerhamii* have optimum pH and temperature at 5 and 32 °C, respectively. These results indicate that the pH and optimum temperature

of polygalacturonase produced by *Kluyveromyces marxianus* CCMB 322 were higher than those of polygalacturonase from *Kluyveromyces wickerhamii*.

3.3 Thermal stability of polygalacturonase enzyme

The polygalacturonase secreted by *K. marxianus* CCMB 322 maintained about 93% of its original activity after heating

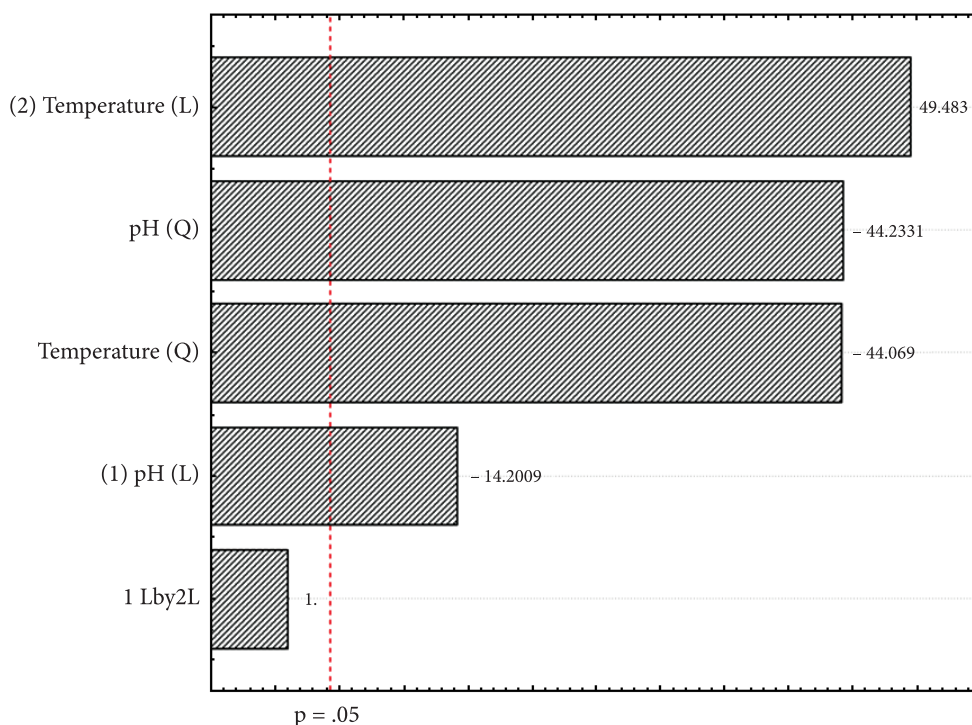


Figure 2. Pareto chart of polygalacturonase activity.

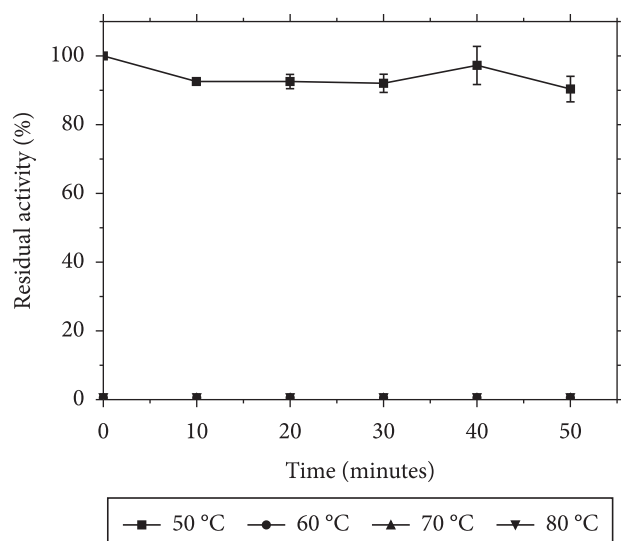


Figure 3. Thermal stability profiles for the pectinase of *Kluyveromyces marxianus* CCMB 322 at 50, 60, 70 and 80 °C.

for 50 minutes at 50 °C in 50 mM acetate buffer (pH 4.5). However, when subjected to temperatures higher than 50 °C, the enzyme probably loses its native structure leading to complete inactivation (Figure 3).

According to Arnold et al. (2001), during the course of evolution, enzymes adjust the strength and number of their stabilizing interactions to optimize the balance between rigidity

(for stability) and flexibility (for activity) at their physiologically relevant temperatures.

Studies of Cordeiro and Martins (2009) showed that the a polygalacturonase from *Bacillus* sp. SMIA-2 retained 70% of activity after heating for 120 minutes at 50 °C.

In several industrial processes such as fruit maceration to extract the juice (60-65 °C), denaturation of the fruit pectin esterase (80 °C), which causes coagulation of the pectin, and pasteurization of juices and musts (90 °C), the material subjected to heating needs to be cooled to 50 °C for treatment with commercial pectinases, which are thermolabile (JAENICKE, 1999). The thermal stability of polygalacturonase from *K. marxianus* CCMB 322 can make this enzyme interesting for use in fruit juice industry.

4 Conclusion

This study demonstrated that *Kluyveromyces marxianus* (CCMB 322) is able to use pectin as a carbon source, although glucose is important in regulating polygalacturonase synthesis, and the product of citrus pectin hydrolysis (galacturonic acid) is the most efficient product for induction.

The polygalacturonase produced by *K. marxianus* CMB 322 showed thermal stability similar to that found in other studies; thus it can hydrolyze the substrate (polygalacturonic acid) at the usual commercial temperature (50 °C), in which there was a reduction of about 7% of the original activity. In addition, the optimum pectinolytic activity was achieved at pH 7.36 and 70 °C (48 hours of incubation).

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