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Production of an endopolygalacturonase from *Wickerhanomyces anomalus* with disintegration activity on plant tissues

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ABSTRACT

The objective of the present study was to produce an endo-polyglacturonase by *Wickerhanomyces anomalus*, in a low cost fermentation medium, by batch and fed-batch cultures in order to be used in the enzyme.

The effect of several nutrients on PGase production, were evaluate at shakes flasks, in a reference fermentation medium, using one factor at a time method, Plackett-Burman design and response surface methodology. The optimized fermentation medium was used to evaluate the growth and production of the enzyme by batch and fed-batch cultures, at a lab scale bioreactor.

PGase activity obtained in the RF medium was ~20 U/mL. The absence of trace element solution had a repressive effect on the enzyme synthesis. The addition of yeast extract, instead of vitamins and amino acids, in the culture medium, improved the production of the enzyme. Plackett-Burman design determined that only pectin and yeast extract had significant and positive effect on PGase production. The Doehlert design determined that maximum PGase synthesis was obtained with 6.0 and 0.8 g/L of pectin and yeast extract, respectively. The final optimized fermentation medium included glucose, pectin, urea, yeast extract and salts. In this medium, PGase synthesis reached ~25 U/mL, and ~49 U/mL, in batch and fed-batch cultures, respectively, at lab scale bioreactor.

In this study, it was able to obtain enzymatic extract with high PGase activity, by the growth of *W. anomalus* in a low cost culture medium, by fed-batch system, for its future use in the enzymatic cassava starch extraction.

1. Introduction

Enzymatic preparations that disintegrate plant tissues are used in the processing of fruits and vegetables. They are traditionally classified into two types according to extent of disintegration (Rombouts and Pilnik, 1980). One type contains those enzymes that can totally disintegrate plant tissues. They are used mainly in production of foodstuffs with high proportions of soluble solids like tomato paste or puree, and also to improve yields in fruit juice production. Usually, a combination of cellulolytic and pectolytic enzymes is needed to achieve almost complete liquefaction. The other type includes the macerating enzymes, which can produce a suspension of loose single cells and are used to prepare fruit nectar bases, vegetable purees, and baby and geriatric foods (Cavalitto and Mignone, 2007; Rojas et al., 2008, 2011; Chen et al., 2014). For such purposes, only the intercellular cementing material that holds together cells and some portion of primary plant cell walls should be removed without damage to adjacent secondary cell walls (to help avoid cell lysis). For this reason, cellulases in the enzyme mixture are undesirable (Nakamura et al., 1995).

Enzymes that degrade pectic substances, which contribute to the firmness and structure of plant tissues, are known as pectinolytic enzymes or pectinases. They constitute a heterogeneous group of enzymes that includes polygalacturonases (PGase), pectin esterases (PE), and pectin lyases (PL) (Jayani et al., 2005; Tari et al., 2007; Rehman et al., 2012). In our previous studies on native microorganism from Misiones Province (Argentina), we isolated a pectinolytic yeast strain (*Wickerhanomyces anomalus*) which produces an extracellular PGase with disintegrating activity on plant tissues (Martos et al., 2013a). Analysis of the culture supernatants revealed only a single enzyme activity, namely endo-PGase (EC 3.2.1.15). This enzyme was purified and

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characterized (Martos et al., 2013b, 2014). PGase from *W. anomalus* is able to macerate several plant tissues (potato, carrot, apple, red pepper, etc.) with the concomitant production of a cell suspension with a high proportion of intact single cells (Zubreski, 2013). A different result was observed when this enzyme was assayed against cassava tuber tissue. In this case, the tissue was completely disintegrated and the starch granules contained within the cells were released into the reaction medium. Microscopic evaluation of the starch grains revealed that they were intact without any mechanical damage. Integrity of starch grains is a very important characteristic that influences the functional properties and, thus, the commercial price of starch. In the conventional process, cassava starch is produced primarily by the wet milling of fresh cassava roots. A substantial amount of starch grains is damaged particularly during the rasping/pulping/grating processes. In addition, these mechanical steps are critical to the economy of the process due to the high energy consumption (Cobana and Antezana, 2007; Torre et al., 2010; Brousse et al., 2012). Therefore, the production of cassava starch including an enzymatic step seems to be an interesting alternative in order increase the quality of the starch obtained and also to reduce the energy consumption of the process. These interesting properties of PGase from *W. anomalus* prompted us to analyze the different factors affecting its production considering a potential application at an industrial level.

2. Material and methods

2.1. Microorganism

W. anomalus, a yeast isolated from citrus fruit peels in the Province of Misiones, Argentine (Martos et al., 2013a), was used.

2.2. Culture media

Yeast medium (g/L): yeast extract (Sigma Chemical Co., St. Louis, Mo, USA), 5; tryptone (Difco-Becton Dickinson & Co., Sparks, MD, USA), 5; glucose (Britania, Buenos Aires, Argentina), 10; agar (Britania), 15; pH 5.0.

Reference fermentation (RF) medium (g/L): glucose (Britania), 10; citrus pectin (Parafarm, Buenos Aires, Argentina), 5; urea, 1,4; KH_2PO_4, 1; $MgSO_4$, 0.5; $CaCl_2$, 0.1; vitamin solution (1000 ×), 1 mL/L; amino acid solution (100 ×), 10 mL/L; trace element solution (1000 ×), 1 mL/L; pH 5.0 (Cavalitto et al., 2000).

Vitamin solution (1000 ×) (Sigma) (μg/L): biotin, 2; calcium pantothenate, 400; folic acid, 2; *myo*-inositol, 2000; niacin, 400; *p*-aminobenzoic acid, 200; pyridoxine, 400; riboflavin, 200; thiamine, 400.

Amino acid solution (100 ×) (Sigma) (mg/L): histidine, 10; methionine, 20; tryptophan, 20.

Trace element solution (1000 ×) (μg/L): $CuSO_4{\cdot}5H_2O$, 40; $FeCl_3{\cdot}6H_2O$, 200; $MnSO_4{\cdot}H_2O$, 400; $NaMoO_4{\cdot}2H_2O$, 200; $ZnSO_4{\cdot}7H_2O$, 400.

Distilled water was used for culture media preparation. Citrus pectin was washed with a 70% (v/v) ethanol-HCl solution (0.05 N) to remove soluble sugars. All components of the medium were autoclaved (121 °C, 15 min), except in the case of vitamins and urea, which were sterilized separately by filtration through a cellulosic filter membrane (0.22 μm, Sartorius).

2.3. Batch cultures in flasks

2.3.1. Enzyme production

Two hundred and fifty milliliter Erlenmeyer flasks with 45 mL of RF medium were inoculated with 5.0 mL of an appropriate dilution of a suspension of the microorganism (OD_{620} = 0.96) grown in yeast medium (30 °C, 24 h). The Erlenmeyer flasks were incubated at 30 °C, for 16 h, in a rotary shaker incubator (MRC, TOU-50N, 25 mm shaking width) at 180 rpm. The biomass was separated by centrifugation at 2350 × *g*, at 5 °C, for 10 min, washed carefully with sterile distilled water and used to inoculate the subsequent fresh medium. Three precultures were performed. In the fourth culture and at the end of the fermentation process, DO_{620} and pH were measured. Then, the culture was centrifuged and the supernatant (enzymatic extract, EE) was frozen for further analytical assays. All experiments were carried out in triplicate and their mean values were used.

2.3.2. Effect of nutritional components on PGase production by one factor at a time method

Preliminary studies were carried out to evaluate the effect of some components of the RF medium on PGase production by *W. anomalus*, using "one factor at a time" method (Rehman et al., 2012). The fermentations were carried out as mentioned above, and experiment runs in the original culture medium (RF) were considered as control.

2.3.3. Effect of trace elements

The strain was cultivated in RF medium without the addition of the trace element solution. Then, in order to know which element(s) has/have a beneficial or an adverse effect on the enzyme expression, other cultures were carried out with RF medium but omitting the addition of one element in each experiment, from the original trace element solution (Ferreyra et al., 2002). For this purpose, five culture media denominated RF_1 to RF_5 were prepared (Table 1).

2.3.4. Effect of the addition of yeast extract

The effect of yeast extract (YE) on PGase production by *W. anomalus* was studied. Cultures were performed in RF medium, with different concentrations of YE (0.1, 0.5 or 0.8 g/L), instead of vitamins and amino acids.

2.4. Experimental design and statistical analysis

2.4.1. Plackett–Burman design

Seven independent variables were selected to evaluate their effect on PGase production by *W. anomalus*, using the mathematical method of Plackett-Burman (PB) in the screening design (Cavalitto and Mignone, 2007; Shabbiri et al., 2012; Brzozowski, 2014). The parameters selected were analyzed at two levels: high (+1) and low (−1). Based on the PB design, the corresponding matrix was obtained. The statistical software Statgraphics Centurión XV was used for analyzing the experimental data.

2.4.2. Response-surface methodology

The significant factors of the PB design were further optimized using response surface method (RSM) (Cheng et al., 2012). A design proposed by Doehlert was selected for studying the response surface (Doehlert, 1970; Butiuk et al., 2015). The real values of the independent variables were coded based on a linear functionality between codified (Z) and actual values (X) according to:

$$X = Z \times \frac{\Delta X}{\Delta Z} + X_o$$

where X_0 is the real value of the central point and ΔX and ΔZ are the

Table 1
Trace elements for RF_1 to RF_5 media.

Trace element	Culture medium					
	RF[a]	RF_1	RF_2	RF_3	RF_4	RF_5
Mo	+	–	+	+	+	+
Fe	+	+	–	+	+	+
Zn	+	+	+	–	+	+
Cu	+	+	+	+	–	+
Mn	+	+	+	+	+	–

[a] Reference fermentation medium.

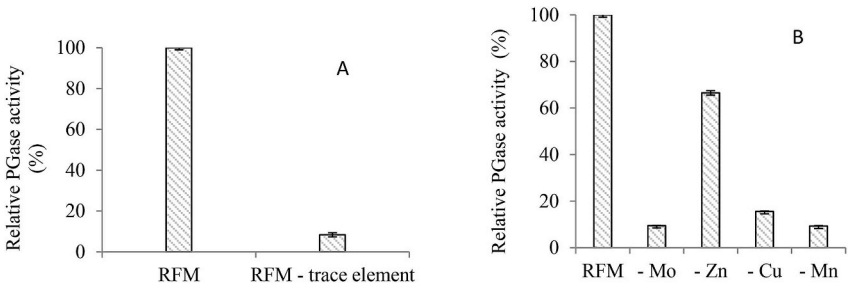

Fig. 1. Effect of the omission of trace element solution (A) andthe omission of one trace element (B), in RF medium, on PGase production by *W. anomalus*.

difference between the highest and lowest values of real and coded numbers, respectively.

For simplicity, a full quadratic model containing six coefficients was used to describe the response observed.

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_{ii}^2 + \sum b_{ij} x_j$$

where Y is the response variable (PGase), b_0 a constant, and b_i, b_{ii}, and b_{ij} are the coefficients for the linear, quadratic and interaction effect, respectively. The number of experiments required in this design (N) is given by $N = n^2 + n + n_0$, where n is the number of variables and n_0 is the number of central points. Replicates at the central level of the variables were performed in order to have an estimation of the experimental variance.

2.5. Validation of the experimental model

To validate the model, additional runs were carried out for PGase production in shake flasks containing the optimized fermentation (OF) medium, predicted by the RSM (Shabbiri et al., 2012; Uzuner and Cekmecelioglu, 2015). Fermentations were conducted in triplicate and their mean values were determined.

2.6. Bioreactor cultures

The kinetics of growth and PGase production of *W. anomalus* in batch and fed-batch cultures were carried out in a 5 L bioreactor (New Brunswick), at 30 °C, with aeration (2.82 L/min) and agitation (500 rpm).

2.6.1. Batch culture

Batch culture was carried out in the OF medium (3 L) and was inoculated with a suspension of the microorganism, grown in Erlenmeyer flasks containing the same medium, at 30 °C, for 24 h. Samples were collected, at suitable periods of incubation (1 h) during 12 h, and centrifuged. Biomass was determined by dry weight and the EE, free of cells, were frozen for further analytical assays.

2.6.2. Fed-batch culture

Fed-batch fermentation was started with a batch culture in OF medium, as it was previously described, and when glucose was depleted, the feeding medium was added into the fermenter at a constant flux rate (F).

In order to control the feeding rate, glucose concentration in the feeding medium (S_R) and F value were estimated with the following equations derived from mass balances for the substrate and biomass in carbon limited cultures by means of the kinetic and stoichiometric parameters calculated in the batch culture (Rojas et al., 2011).

$$S_R = \frac{X_f \cdot V_f - X_0 \cdot V_0}{Y_{x/s} \cdot (V_f - V_0)} \quad (1)$$

$$F = \frac{\mu_0 \cdot V_0 \cdot X_0}{S_R \cdot Y_{X/S}} \quad (2)$$

where S_R is the concentration of the growth limiting substrate in the feeding medium; X_o and X_f, are the biomass concentrations at the beginning and the end of the feeding phase (g/L), respectively; V_0 and V_f the initial and final volumes (L), respectively; F the feeding rate (mL/h); μ_0 the initial specific growth rate (h^{-1}) and $Y_{x/s}$, cellular yield coefficient based on carbon source consumption ($g_{cell}/g_{carbon\ source}$).

Concentrated OF medium (5 ×) without the addition of citrus pectin and with the corresponding glucose concentration (S_R) was the feeding medium. Samples were collected at suitable periods of incubation and it was determined biomass, residual glucose and PGase activity.

2.7. Analytical methods

Glucose: was determined using the glucose-peroxidase (Glicemia, Wiener, Argentina) method.

PGase activity: was assayed by measuring the reducing groups released from 2 g/L polygalacturonic acid (Sigma) solution, in sodium acetate/acetic acid buffer (0.2 M, pH 5.0), using Somogyi-Nelson method. The reaction was carried out at 37 °C for 10 min. A calibration curve was made using galacturonic acid (GA) as standard. One unit of PGase was defined as the amount of enzyme that releases 1 μmol of GA per minute (Herbert et al., 1971.; Cavalitto et al., 2000; Ferreyra et al., 2002).

3. Results and discussion

3.1. Batch cultures in flasks

3.1.1. Effect of nutritional components on PGase production by one factor at a time method

Fig. 1 shows the production of PGase by *W. anomalus* in RF medium without the addition of trace elements (A) and omitting the addition of one element at a time (B). Fig. 2 shows PGase production in the RF medium with different concentrations of YE but omitting the addition of amino acid and vitamin solutions. In these figures, the results are presented as percentage of the value attained with RF medium (19.57 ± 0.55 U/mL) and this value corresponds to 100% of the

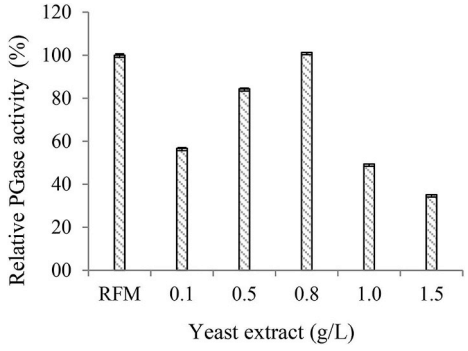

Fig. 2. Effect of yeast extract on PGase production by *W. anomalus*.

enzyme activity.

The pH of the medium remained almost constant (close to 4.0) during the whole process. Urea is hydrolyzed by urease positive microorganisms (such as *W. anomalus*), releasing ammonia and carbon dioxide. As long as there is carbon and energy source in the medium, the ammonia is assimilated to the biomass and therefore the pH is maintained at a constant value (Rojas et al., 2008).

The absence of trace element solution in RF medium had a repressive effect on the enzyme synthesis. A decrease of 93% was observed with respect to the value obtained in the original RF medium (Fig. 1). The absence of Mn, Mo and Cu decreased PGase production by 93.7, 93.5 and 81.4%, respectively. The repressor effect was lower in the absence of Zn, with a decrease of 54.8% (Fig. 2). This clearly demonstrates that trace elements in the culture medium are needed for good enzyme production.

Cavalitto and Mignone (2007) studied the effect of Ca, Mg, Mo, I, B, Co, Mn, Zn, Fe, Cu and K_2HPO_4, on protopectinase production by *G. klebahnii*, a yeast-like microorganism, and concluded that only Fe had a positive effect on enzyme production.

Fig. 2 shows that as the concentration of YE in the culture medium increased from 0.1 to 0.8 g/L, a higher PGase production was observed, but above this concentration, the enzyme synthesis decreased.

PGase production by *Bacillus licheniformis* KIBGE IB-21 increased with an increased in YE concentration and maximum production was achieved when 0.3% was incorporated into the medium (Rehman et al., 2012).

3.2. Experimental design and statistical analysis

3.2.1. Plackett–Burman design (PBD)

Seven independent variables were selected to evaluate their effect on PGase production by *W. anomalus*, using the mathematical method of Plackett-Burman (PB) screening design. The variables studied were: pectin, KH_2PO_4, $MgSO_4$, $CaCl_2$, $FeCl_3$, YE and trace elements. The cultures were performed in the RF medium omitting the addition of amino acid and vitamin solutions. Variables were analyzed at two levels: high (+1) and low (−1) (Table 2). The matrix developed by the PB design and the results (PGase activity) are presented in Table 3, the regression analysis is shown in Table 4 and in Fig. 3 the Pareto chart.

Statistical analysis of the data (Table 4) showed that only pectin, yeast extract and Ca, had significant effect on PGase production ($p < 0.05$). The R^2 was 0.934, indicating that 93.4% of the variability in the response could be explained by the model.

Pareto chart (Fig. 3) indicates that pectin and yeast extract had a positive effect on PGase production. Ca had a negative influence, which means that a reduction in the concentration of this element in the culture medium will enhance the enzyme synthesis. In the presence of yeast extract, the influence of trace elements was not significant, because it is also a mineral source (Rehman et al., 2012).

The effect of salts on enzyme production has been subject of different publications. Cheng et al. (2012) evaluated the effect of NH_4Cl, $MgSO_4$, KH_2PO_4, $FeSO_4$, $ZnSO_4$, $CaCl_2$, $MnSO_4$ and NaCl on chitosanase production by *S. albus* YT2 and reported that only $MgSO_4$ had a significant effect. Mahesh et al. (2014) determined that $MgSO_4$ and $CuSO_4$ were responsible to enhance the production of an endo-PGase by *A. niger* MTCC 3323; KH_2PO_4 showed less effect and the remaining factors such as $CaCl_2$, $ZnSO_4$, $FeSO_4$, $MnSO_4$ and K_2HPO_4 showed no significant effect. According to Paudel et al. (2015), the production of PGase by *Bacillus* sp HD2 decreased with increasing Ca concentration in the culture medium.

Table 2
Factors, codes and real levels used in the PB design.

Variable (g/L)	Code	Level of variable	
		(+1)	(-1)
Pectin	A	10.0	2.5
YE	B	0.1	1.5
Trace element solution	C	0.0005	0
$FeCl_3$	D	0.0002	0.0001
$CaCl_2$,	E	0.1	0.05
$MgSO_4$	F	0.5	0.25
K_2HPO_4	G	1	0.5

Table 3
Experimental Plackett-Burman matrix and PGase activity obtained.

Exp.	Variable[a]							PGase[b] (U/mL)
	A	B	C	D	E	F	G	
1	+1	+1	+1	−1	+1	+1	−1	20.447
2	+1	+1	−1	+1	−1	−1	−1	20.839
3	−1	−1	−1	+1	+1	+1	−1	6.616
4	+1	−1	−1	−1	+1	+1	+1	17.184
5	+1	+1	−1	+1	+1	−1	+1	16.098
6	−1	+1	+1	+1	−1	+1	+1	18.440
7	+1	−1	+1	−1	−1	−1	+1	20.632
8	−1	−1	+1	−1	−1	−1	−1	11.921
9	−1	−1	−1	−1	−1	−1	−1	14.942
10	+1	−1	+1	+1	−1	+1	−1	13.899
11	−1	+1	−1	−1	−1	+1	+1	19.540
12	−1	−1	+1	+1	+1	−1	+1	9.658

[a] Pectin (A), yeast extract (B), trace element solution (C), Fe^{+2} (D), Ca^{+2} (E), Mg^{+2} (F), K_2HPO_4 (G).
[b] Mean of three values, SD within 10%.

Table 4
Analysis of variance (ANOVA) for the experimental parameters of Plackett-Burman design.

Variable	SS	DF	MS	*F*-value	p-value
A	65.249	1	65.249	14.94	0.018*
B	49.426	1	49.426	11.32	0.028*
C	0.004	1	0.0041	0	0.977**
D	30.452	1	30.452	6.97	0.057**
E	57.939	1	57.939	13.26	0.022*
F	0.345	1	0.3454	0.08	0.793**
G	13.842	1	13.842	3.17	0.149**
Error	17.473	4	4.3682		
Corrected total	234.731	11			

* Significant values ($p < 0.05$).
** Non-significant values ($p > 0.05$).
SS: Sum of squares; DF: degrees of freedom; MS: mean square. R^2: 0.934.

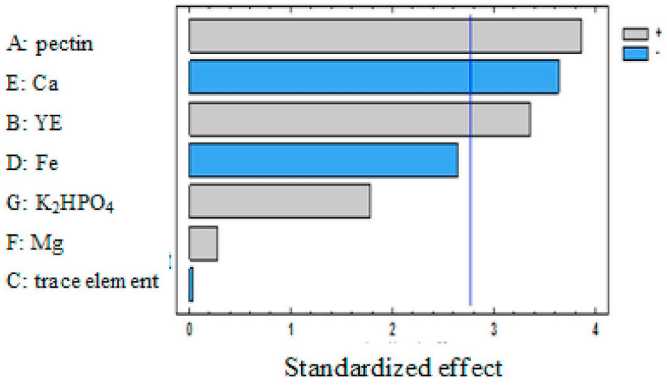

Fig. 3. Pareto chart of Plackett Burman design.

3.2.2. Response-surface methodology (RSM)

According to the results of the PB design, the significant factors with positive effect (pectin and YE), were optimized using response surface

Table 5
Codified, actual values and enzyme activity attained in the Doehlert design.

Exp.	Codified value		Actual value (g/L)		PGase activity[a] (U/mL)
	YE	Pectin	YE	Pectin	
1	1	0	1.5	6.25	9.739
2	0.5	−0.866	1.15	10	13.735
3	−0.5	−0.866	0.45	10	10.129
4	−1	0	0.1	6.25	14.813
5	−0.5	0.866	0.45	2.5	11.352
6	0.5	0.866	1.15	2.5	11.573
7	0	0	0.8	6.25	22.318
8	0	0	0.8	6.25	22.527
9	0	0	0.8	6.25	22.641

[a] Mean of three values, SD within 10%. YE: yeast extract.

method. The other variables were maintained in the basal level. The Doehlert experimental design was applied. The number of central point value (n_0) was fixed at 3 and with two factors, the total of points of Doehlert matrix was 9. The matrix of the Doehlert design and the results of enzyme activity obtained in each experiment are shown in Table 5.

Data in Table 5 were converted into second order polynomial equation. Values of linear, quadratic, and cross coefficients and their level of significance (calculated by ANOVA) are given in Table 6.

$$PGase\left(\frac{UE}{mL}\right) = -16.2 + 29.97 * YE + 9.08 * Pectin - 17.86 * YE^2 - 0.44 * YE * Pectin - 0.72 * Pectin^2 \quad (3)$$

The statistical analysis showed that both, pectin and YE concentration, as well as their interactions, had a significant effect on PGase production, in the range studied. The regression coefficient ($R^2 = 0.99$) indicated that the model was able to explain more than 99% of the observed response. It was also observed that the most important factor impacting PGase synthesis was YE, with the highest coefficient (29.97), followed by pectin (9.08). The negative values of quadratic effects for both variables indicated the existence of a maximum as a function of these variables (Table 6).

Fig. 5 shows the response surfaces for PGase production after 16 h of culture, as a function of the two independent variables. An increase in PGase production was observed when pectin increased up to ~6 g/L and YE up to ~0.8 g/L; above these values, the enzyme activity declined (Fig. 4). The second order polynomial equation determined a maximum PGase value of 23.54 ± 0.43 U/mL, in the medium containing 6 g/L of citrus pectin and 0.75 g/L of YE (Eq. (3)).

A low initial pectin concentration results in a low enzyme production, since *W. anomalus* PGase is an inducible enzyme (Martos et al., 2013a). The reduction in PGase production observed at high pectin concentrations might be ascribed to the increase in the viscosity of the culture medium, which caused problems to maintain the homogeneity and in oxygen transfer (Rehman et al., 2012).

YE contains essential vitamins, minerals and amino acids, which are necessary for microorganism growth and enzyme production (Rehman et al., 2012; Paudel et al., 2015). However, it also contains some strong chelating agents, and it is possible that these compounds may have, at high concentration, a deleterious effects on enzyme expression (Ferreyra et al., 2002). A similar nonlinear effect of YE on PGase production was reported by *Bacillus* HD2 (Paudel et al., 2015) and *Bacillus subtilis* (Uzuner and Cekmecelioglu, 2015) and for the production of phytase by *P. anomala* (Kaur and Satyanarayana, 2005).

The final composition of the Optimized Fermentation (OF) medium is shown in Table 7.

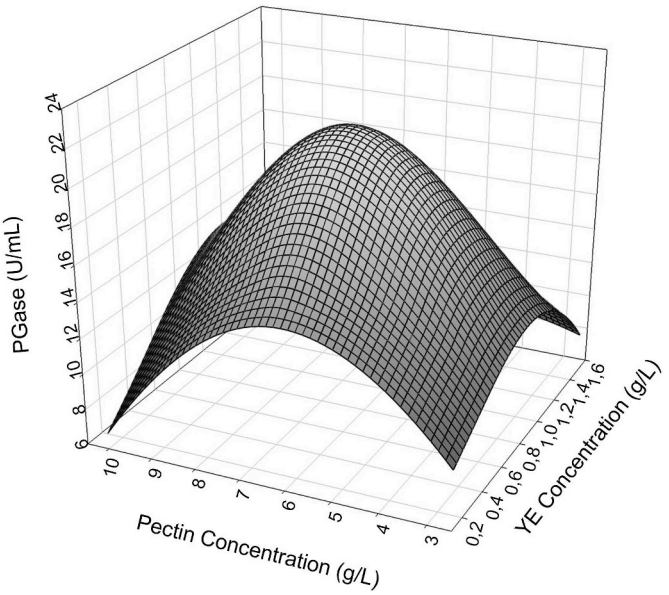

Fig. 4. Response surface curve of PGase production as a function of pectin and yeast extract.

3.3. Validation of the experimental model

The enzyme production attained in shake flasks containing the OF medium predicted by RSM was 23.2 ± 0.78 U/mL, after 16 h of culture. Analysis of variance revealed no significant differences ($p < 0.05$) in PGase activity between predicted and experimental values, showing the validity of the model used.

3.4. Bioreactor cultures

3.4.1. Batch culture

Fig. 5 shows the time course of a batch culture of *W. anomalus* in OF medium, at a laboratory scale bioreactor. The microorganism grew in exponential phase until 10 h of culture. Glucose was exhausted when the culture reached the stationary growth phase. The biomass produced led to a biomass yield ($Y_{x/s}$) of 0.30 g_x/g_s (g of cell dry weight per gram of glucose consumed). A maximal specific growth rate (μ_m) of 0.164 h^{-1} (R^2: 0.92) was calculated. PG synthesis appears to be associated with yeast growth, reaching 25.52 ± 0.147 U/mL at the end of the

Table 6
Significance level and value of the coefficients, calculated by ANOVA, in the full quadratic model obtained according to Doehlert design.

	Coefficient	P
Constant	−16.20	0.00040
YE	29.97	0.00000
Pectin	9.08	0.00000
YE^2	−17.86	0.00260
YE . pectin	−0.44	0.00000
$Pectin^2$	−0.72	0.00000

$R^2 = 0.99$.

Table 7
Composition of the optimized fermentation medium.

Component	(g/L)
Glucose	10
Pectin	6
Urea	1.4
YE	0.75
KH_2PO_4	0.5
$CaCl_2$	0.05
$MgSO_4$	0.25

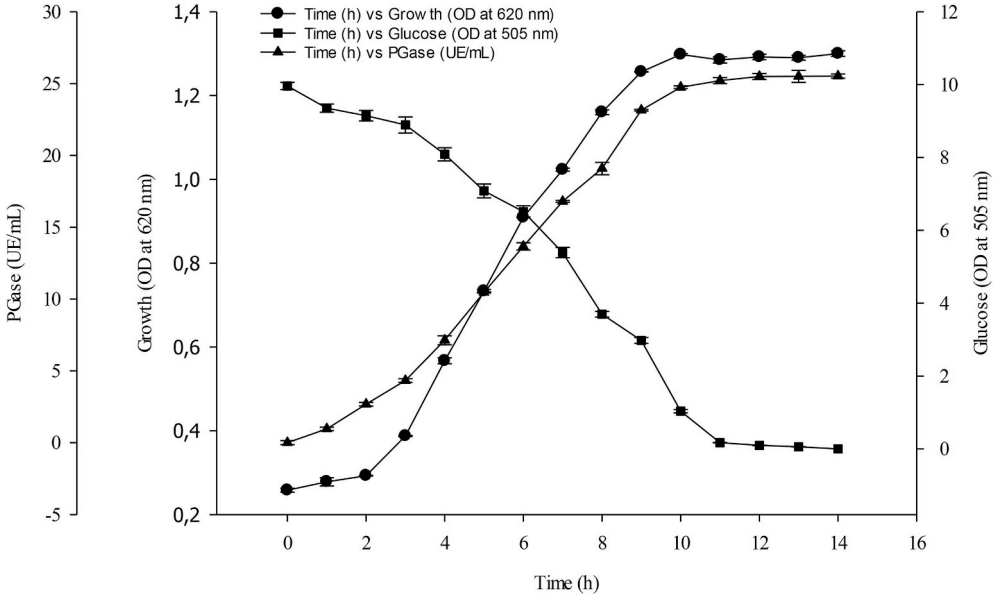

Fig. 5. Time course of biomass, enzyme activity and residual glucose in a batch culture of *W. anomalus*.

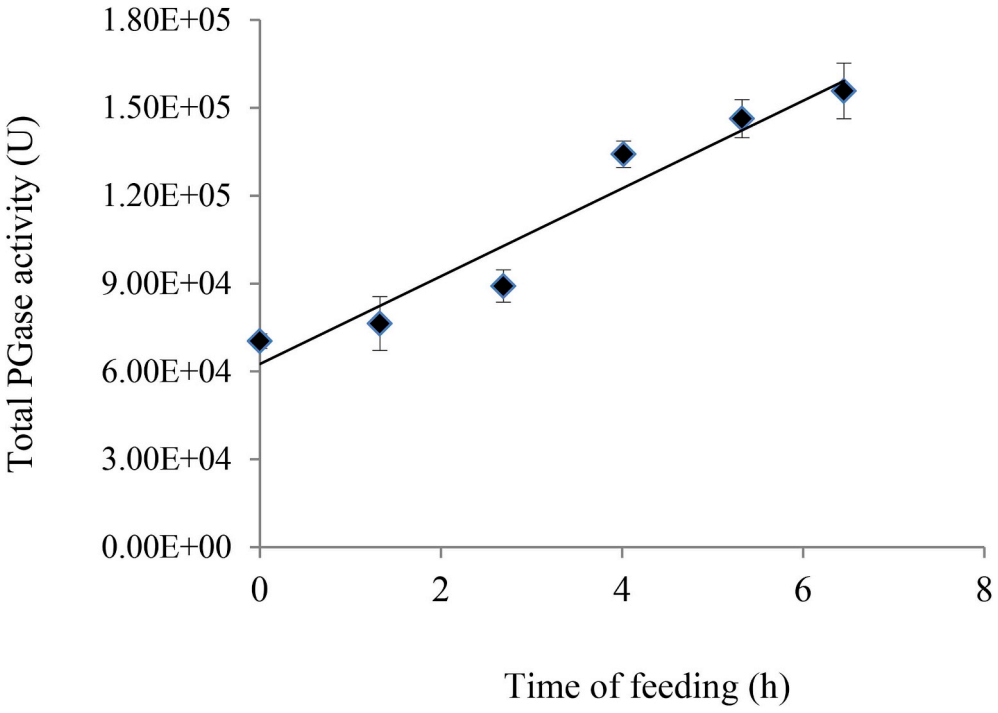

Fig. 6. Time course of total PGase activity during fed-batch culture of *W. anomalus*.

culture. The pH remained at values close to 4.0 in the course of the process.

Endo-PGase produced by this wild yeast strain, was higher than that reported by others authors (using the same quantification technique for PGase activity). Contreras Esquivel et al. (1999) reported that *A. kawachii* produced 65 mU/mL of an acidic PPase, which was associated with fungal growth. It was reported values of 10–11 U/mL for the production of an endo-PGase with pectin releasing activity by *Geotrichum klebanii* ATCC 42397 in a defined medium based on urea, glucose, mineral salts and vitamins (Cavalitto et al., 2000). Tari et al. (2007) reports that *A. sojae* ATCC 20235 grown in a liquid medium with glucose, peptone, salts and maltodextrin, produced a maximum PGase activity of 13.5 U/ml. Rojas et al. (2011) reported that a recombinant *S. cerevisiae* which expressed the acidic PPase from *A. kawachii*, produced in a batch culture 3 U/mL of the enzyme after the induction with galactose.

3.4.2. Fed batch culture in a lab scale bioreactor

The corresponding parameters used to model the fed-batch culture were determined from the batch culture data: X_o = 3.0 g/L, V_o = 3 L; $Y_{x/s}$ = 0.30 g_x/g_s. The values of V_f, X_f and μ_0, were arbitrary chosen for the experiment: X_f = 6 g_x/L, V_f, = 3.5 L, and μ_0 = 0.160 h^{-1} ($< \mu_m$). The design equations (Eqs. (1) and (2)) determined: F = 60 mL/h and S_R = 80 g_s/L. Time course of enzyme production is shown in Fig. 6.

A constant biomass accumulation rate (X.V) was observed, and glucose concentration in culture medium was negligible during the whole process, in good accordance with a fed-batch culture at constant flux. PG activity increased with feeding time, reaching at the end of the

culture a value of 48.93 ± 0.38 U/mL, corresponding to a productivity of 3.19 U/mL. This enzyme activity was higher than that obtained in batch culture. The reason for the improved PGase productivity was the greater biomass concentration achieved in this culture system, considering that PGase is growth associated (Cavalitto et al., 2000). These results are in accordance with those reported by others authors (Gummadi and Kumar, 2008; Rojas et al., 2010, 2011).

4. Conclusions

In this study, it was able to obtain, a low cost and simple to prepare culture medium, for production of PGase from *W. anomalus*, by using PB design followed by RSM, appropriate for production of PGase by *W. anomalus*, at large scale. The optimized fermentation medium included glucose, pectin, urea, yeast extract, KH_2PO_4, $CaCl_2$, $MgSO_4$, in which the amino acids, vitamins and trace elements, were eliminated from the original one. In this medium, PGase synthesis reached ~25.52 U/mL, after 10 h, in batch system, at a lab scale bioreactor. In fed-batch system, PG activity increased with feeding time, reaching at the end of the culture a value of ~49 U/mL.

Therefore, fed-batch culture using the optimized fermentation medium could be an adequate alternative for industrial production of PGase by *W. anomalus*, enzyme of interest in different food processes, but particularly for the bioprocessing of cassava roots for starch production.

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