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Influence of nutritional and environmental factors on ethanol and endopolygalacturonase co-production by *Kluyveromyces marxianus* CCEBI 2011

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Summary. Ethanol and endopolygalacturonase (endoPG) are simultaneously produced by the yeast *Kluyveromyces marxianus* CCEBI 2011. The aim of this study was to determine the optimal combination of seven environmental and nutritional variables, as well as the influence of each one, with respect to the fermentation process in yeast cultures in which sugarcane juice was the substrate. Simplex sequential optimization showed that after 15 runs the optimal conditions were: pH, 4.6; temperature, 31ºC; total reducing sugars (TRS), 125 g/l; $(NH_4)_2SO_4$, 2.48 g/l; $(NH_4)_2HPO_4$, 2.73 g/l; $CaCl_2$, 0.33 g/l and $MgSO_4 \cdot 7H_2O$, 0.54 g/l. Under these conditions, the ethanol concentration was 47.6 g/l and endoPG concentration was 9.8 U/ml, which represented increases of 22% and 10%, respectively, over the concentrations obtained under suboptimal conditions. Temperature and $(NH_4)_2SO_4$ supplementation were the most significant factors influencing the co-production process. [**Int Microbiol** 2011; 14(1):41-49]

Keywords: *Kluyveromyces marxianus* · ethanol · polygalacturonase · sugarcane juice · optimization · Simplex method

Introduction

Pectinases comprise several enzymes that promote the natural degradation of pectins, present in plant cell walls. Pectin is a complex polymer constituted by a backbone of (1→4)-α-D-galacturonic acid residues, partially methyl-esterified, which is responsible for the cohesiveness and integrity of plant tissues [11]. The presence of pectic substances causes problems in the elaboration of fruit juices, particularly during the clarification and filtration steps. Pectinases are classified into esterases, which de-esterify the pectin molecule without altering the degree of polymerization, and depolymerases, which split the galacturonate backbone. The depolymerizing enzymes are futher grouped into polygalacturonases (PG), which cleave glycosidic bonds by hydrolysis, and pectin-lyases, which break the glycosidic linkages by β-elimination. Pectin-lyases and PG are sub-classified as *endo* or *exo*, according to whether their mechanism of attack is random or terminal, respectively [17].

Pectic enzymes are widely used in industry, with applications such as the extraction and clarification of fruit juices,

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the production of fermentable sugars from plant biomass [6], oil and pigment extraction, and the processing of textile fibers [12]. They are also used in formulations of animal feed [9], contributing to better nutrient assimilation. EndoPG form an industrially important subgroup of pectinases, because they promote a rapid decrease in the molecular weight of pectin and hence in the viscosity of pectin-containing solutions, with minimum release of reducing sugars.

Currently, one of the main sources of pectic enzymes for industrial use is the mold *Aspergillus niger*. Commercial preparations consist of a mixture of different pectic enzymes (endo- and exo-PG, pectin lyases, and pectin esterases) but also enzymes with undesirable side effects [9,24,37]. Accordingly, new sources of pectic enzymes have been evaluated. In this regard, some ascomycetous yeasts, such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, which only produce endoPG, provide an interesting alternative [7]. However, the relatively low yield of these enzymes in wild-type strains of these yeasts represents a drawback to their industrial production and has spurred the cloning and expression of the respective genes from *S. cerevisiae* [9].

In a previous work, we showed that during alcoholic fermentation by *K. marxianus* CCEBI 2011, endoPG accumulate together with ethanol in the culture broth [34], which contributes favorably to the global economy of the bioprocess. Thus, it was of interest to explore the substrates and fermentation conditions allowing optimization of the process. Knowledge of yeast growth under oxic conditions is important to establish the optimal conditions for the propagation of these microorganisms and allowing high biomass yield and cell viability to be reached in a short time, thus satisfying industrial production requirements.

Alcohol production in Cuba is based on sugarcane molasses as the fermentation substrate. However, the black color of molasses results in difficulties in the recuperation or use of other fermentation products, such as endoPG, which cannot be distilled. Therefore, an alternative and renewable carbon source is required, among which sugarcane juice seems to be the most promising, based on its light yellow color and 12–17% total sugars, of which 90% is sucrose and 10% is glucose plus fructose [36]. Moreover, sugarcane juice contains organic nutrients and minerals, both in amounts suitable for ethanol production by fermentation [25,27]. In the present work, we used Simplex sequential optimization to determine the conditions yielding the optimal co-production of ethanol and endoPG by *K. marxianus* CCEBI 2011 from sugarcane juice. The proportional influence of the studied environmental and nutritional variables on this bioprocess was also established.

Materials and methods

Microorganism and inoculum. *Kluyveromyces marxianus* CCEBI 2011 strain was obtained from the Industrial Biotechnology Studies Center, University of Oriente (Santiago de Cuba, Cuba). The yeast culture was kept on yeast extract-peptone-glucose (YPG) slants at 4ºC and subcultured at 15–21 days intervals. Propagation medium for inocula was formulated as follows: clarified sugarcane juice, prepared so as to reach a content of total reducing sugars (TRS) of 20 g/l; $(NH_4)_2SO_4$, 2 g/l and $(NH_4)_2HPO_4$, 2 g/l. A 200-ml conical flask containing 40 ml of this medium was inoculated with a single loopful of a 24-h yeast culture from YPG plates. The culture was then incubated at 30ºC in an orbital shaker at 200 rpm for 10 h, after which the growing culture was transferred (1% v/v) into a 500-ml conical flask containing 100 ml of propagation medium, and incubated for an additional 8.5 h under the same conditions. This culture was used as the inoculum in subsequent experiments.

Sugarcane juice and media for alcoholic fermentations. The sugarcane juice was collected at the "Dos Ríos" sugarcane factory in Santiago de Cuba, and was clarified and stored at –20ºC. Clarification was achieved by adding $Ca(H_2PO_4)_2$ until 300 mg of P_2O_5 per liter of juice was reached, followed by heating at 80ºC for 20 min. The solution was then cooled at room temperature and finally centrifuged at 3000 rpm for 15 min to remove the sedimented solids. The main characteristics of the clarified sugarcane juice are shown in Table 1.

Kinetics of *K. marxianus* growth in sugarcane juice under aerobic batch culture. During propagation in a 500-ml conical flask, aliquots were collected every hour over the first 8 h, and then at 30 min intervals up to 14 h. Biomass was measured spectrophotometrically. The specific growth rate was calculated by plotting the log of the biomass versus time.

Table 1. Some of the main characteristics of the clarified sugarcane juice used in the culture medium

Characteristics	Average measured value
pH[a]	6.0
Total acidity (% w/w)[a,b]	0.087
Soluble solids (ºBrix)[a]	22.6
Sucrose (% w/w)[a]	17.7
Free reducing sugars (% w/w)[a]	0.76
Total reducing sugars (% w/w)[a]	19.41
Soluble proteins (g/l)	0.80
Mg (% w/v)[c]	0.23
Ca (% w/v)[c]	0.13
Absorbance (620 nm)	0.520

[a]Analysis carried out according to ICINAZ (Cuba), 1996.
[b]As acetic acid.
[c]Mg and Ca expressed as MgO and CaO, respectively, on the basis of soluble solids.

Table 2. Experimental trials suggested by the Simplex algorithm

Trial	$[H^+]$ (μM)	Temperature (°C)	TRS (g/l)	$(NH_4)_2SO_4$ (g/l)	$(NH_4)_2HPO_4$ (g/l)	$CaCl_2$ (g/l)	$MgSO_4 \cdot 7H_2O$ (g/l)
1	24	40	106.3	2.25	0.75	0.25	0.25
2	24	28	106.3	0.75	2.25	0.75	0.25
3	8.7	40	68.8	0.75	0.75	0.75	0.25
4*	8.7	28	68.8	2.25	2.25	0.25	0.25
5*	8.7	40	106.3	2.25	2.25	0.75	0.75
6	24	28	68.8	2.25	0.75	0.75	0.75
7	24	40	68.8	0.75	2.25	0.25	0.75
8*	8.7	28	106.3	0.75	0.75	0.25	0.75
9	26.2	42	63.4	2.46	2.46	0.82	0.18
10	7.1	42	110.1	1.02	2.52	0.34	0.02
11*	18.4	29	82.5	1.84	1.20	0.61	0.55
12*	24.8	31	125	2.48	2.73	0.33	0.54
13	31.4	28	88.8	2.63	1.02	0.73	0.91
14*	27	26	78.1	2.63	1.45	0.73	0.91
15*	16.3	35	87.5	3.44	1.49	0.32	0.87
RV	16.3	35	87.5	1.50	1.50	0.50	0.50

$[H^+]$ Hydrogen ion concentration; RV: Reference values used in the first Simplex design.
Trials 1–8 correspond to the first Simplex run. *Trials included in Simplex vertices after 15 runs are marked with asterisks.

Batch alcoholic fermentation. Alcoholic fermentations were performed in 10-ml (98 × 14 mm) tubes containing 2.5 ml of the culture medium inoculated with ca. 3.2×10^7 cells. The cultures were incubated under static conditions at the temperature set for each experiment until a constant TRS concentration was reached. Fermentations and analyses were carried out in triplicate.

Analytical determinations and endoPG activity assays. Clarified sugarcane juice was characterized according to the standard methods used by the Cuban sugar industry. The pH was measured in a Pacitronic (Germany) pH-meter; total acidity was titrated with 0.1 N NaOH and expressed as grams of acetic acid in 100 ml of juice; soluble solids were estimated by refractometry and expressed in ºBrix. Sucrose and total and free reducing sugars were determined at constant volume (75 ml) by means of the titrimetric Eynon-Lane method. Sucrose inversion, in a previous Eynon-Lane titration step, was carried out using Walker´s method. In brief, sugarcane juice was heated in a steam-bath for 30 min in the presence of 0.5 N hydrochloric acid and then immediately neutralized with 5 N NaOH. Soluble protein, magnesium, and calcium contents were also measured. Protein was estimated using the Folin phenol reagent [26], with bovine serum albumin (BDH, England) as standard. Metals were quantified by volumetric analysis from previously acid-mineralized samples. In the cell-free culture broths, ethanol was determined by the Conway colorimetric method [14], and TRS were estimated according to the Somogyi-Nelson procedure [31,35]. Acid hydrolysis according to Walker's method was performed prior to TRS determination. Biomass was indirectly measured spectrophotometrically at a wavelength of 620 nm, with a yeast suspension whose concentration was gravimetrically measured (as dry weight) as standard.

EndoPG activity was assayed by incubating a mixture of 400 μL of 0.5% (w/v) polygalacturonic acid (sodium salt, from Sigma) in 50 mM of sodium acetate buffer, pH 5.0, and 100 μL of enzymatic sample in the same buffer. The enzymatic reaction was carried out at 37ºC for 10 min. EndoPG activity was estimated based on the increase in reducing power and was evaluated using the Somogyi method as modified by Nelson [31,35]. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol/min of galacturonic acid or equivalent reducing power under these conditions.

Simplex optimization. The Simplex method was used to maximize the combined production of ethanol (expressed as volumetric productivity, in g/l·h) and endoPG (expressed in U/ml). The MultiSimplex A.B. software (v. 2.1.1; Grabitech, Sweden, 1988) was used for this purpose. Seven environmental and nutritional variables with a potentially significant influence

on ethanol and endoPG production were taken into account. These variables, their reference values, and the experimental trials suggested by the Simplex algorithm are given in Table 2. Reference values were established on the basis of earlier work [34]. The algorithm settings were: reflection coefficient, 1; negative and positive contraction, –0.5 and 0.5, respectively, and expansion coefficient, 2. The limit values of the response values set to the calculus of the joint response by Multisimplex software were: PG activity, 0 and 10 U/ml; ethanol productivity, 0 and 2 g/l·h. Both cases were considered to have the same level of importance.

Statistical analysis. Experimental data were analyzed by using multiple linear regression to determine the weight of influence of the evaluated environmental and nutritional factors on ethanol and PG production. A 5% level was defined as significant. The program Statgraphics Plus 3.1 (Statistical Graphics Rockville, MD, USA) was used for this purpose.

Results and Discussion

Growth kinetics of *K. marxianus* in sugarcane juice under oxic conditions. *Kluyveromyces marxianus* CCEBI 2011 cultured under oxic conditions in sugarcane-juice-based medium initially entered a short lag phase of approximately 2 h, followed by an exponential phase until 7 h, and then a progressive decrease in growth (7–15 h), during which there were only small variations in the amount of biomass.

The maximum specific growth rate (μ_{max}) estimated during exponential phase was 0.71 ± 0.06 h^{-1}. This μ_{max} value is higher than other reported values for *K. marxianus* strains growing aerobically. Banat et al. [5] reported a value of 0.58–0.63 h^{-1}, and more recently Fonseca et al. [19] and Almeida et al. [1] quoted values of 0.56 h^{-1}. In these cases, glucose was the only carbon source.

The long deceleration phase, observed here after 7 h, was probably related to nutritional limitations. Sugarcane juice contains negligible amounts of vitamins [30] and low levels of potassium, phosphorous, and magnesium [16]. The yeast strain used in this study requires biotin for growth [Serrat M (2003) Ph.D. thesis, University of Oriente, Santiago de Cuba, Cuba], and limiting amounts of this micronutrient may

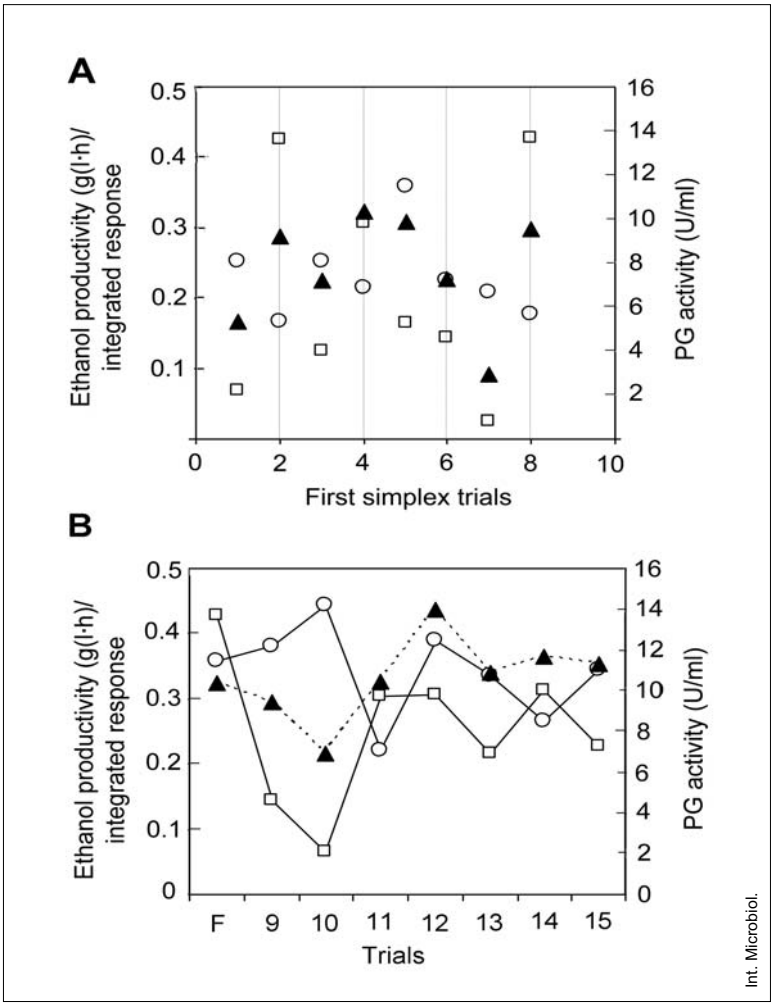

Fig. 1. Behavior of ethanol productivity (circles), PG activity (squares), and joint response measure (triangles) during Simplex optimization. (**A**) First Simplex. (**B**) Sequential optimization, in which the F "trial" shows the best result of each response variable among eight initial trials corresponding to the first Simplex.

account for the results obtained. Da Silva [15] has reported that other microorganisms, such as *Escherichia coli* and *Klebsiella oxytoca*, are unable to grow in sugarcane juice unless it is supplemented with yeast extract, as a source of vitamins.

The biomass yield obtained under these conditions was 0.23 g/g sugar, which is low for aerobic growth when carbohydrates serve as carbon sources. In addition to the above-mentioned nutritional imbalance in sugarcane juice, the energetic cost of sucrose hydrolysis and subsequent uptake by the yeast cells should be considered. Sucrose is rarely found in coffee beans, the natural environment of *K. marxianus* [33].

In view of the characteristics of aerobic growth in sugarcane juice, we decided to use a culture of 8.5 h as the inoculum in the alcoholic fermentation experiments. This time point corresponded to the end of the exponential phase and the beginning of the deceleration phase, when biomass concentration was ca. 1.67 g/l, with a cellular concentration of approximately 1.3×10^8 cell/ml. Therefore, despite the small size of this inoculum, an optimal physiologic state and high cell viability were achieved.

Optimizing the co-production of ethanol and endoPG by *K. marxianus* in sugarcane juice. Optimization of ethanol and PG concomitant production is a complex task, since the conditions that increase the formation of one product reduce the formation of the other, as observed in trials 2 and 8 (high PG activities) and 1 and 5 (high ethanol productivities) (Fig. 1A). When the Simplex method was adjusted to experimental conditions in which a compromise situation would be reached, acceptable high levels of both responses, and thus a maximum combined response, were obtained. By run number 11 and the following trials, the membership function (integrated response) began to show similar values (Fig. 1B), suggesting that the optimization process had arrived at a region where the response could not be further improved. Hence, the experiment was stopped at this point.

Table 3 shows the main fermentation efficiency indicators reached in the optimum region, corresponding to trial 12. Under these optimized conditions, the ethanol concentration was 47.6 g/l, which represents an increase of 22% compared to the concentration obtained in a previous study under sub-optimal conditions [34]. Productivity was 0.39 g/l·h, lower than the recommended values for industrial ethanol production, which range from 1 to 3 g/l·h [13]. This was a direct consequence of the small size of the starter culture utilized in these experiments, which was 0.167 g/l. With a still low, but reasonable starting cell concentration of 1 g/l, a five-fold increase in ethanol productivity (assuming a lineal relation) could be expected. Note that to obtain short-time fermentations, inoculum sizes of 30–40 g/l are necessary [23].

Table 3. Fermentation efficiency indicators under optimized conditions

Variable	Value[a]
Biomass yield	0.020 ± 0.001
Ethanol (g/l)	47.60 ± 1.21
Q_{vol} ethanol (g/l·h)	0.390 ± 0.009
Fermentation efficiency (%)[b]	82.5 ± 2.1
PG activity (U/ml)	9.8 ± 1.0
Q_{vol} PG (U/ml·h)	0.080 ± 0.01
Specific PG production (U/mg)	5.54 ± 1.13

Q_{vol}, volumetric productivity.
[a]Average ± standard deviation.
[b]Expressed as percent of the maximum theoretical yield of ethanol from glucose.

Comparable values were obtained by Hack and Marchant [22] under similar fermentative conditions. Using the *K. marxianus* strain IMB3 in molasses at 45ºC, they obtained a final ethanol concentration of 40 g/l. However, Limtong et al. [25] achieved a productivity value of 1.45 g/l·h and a final ethanol concentration of 87 g/l using *K. marxianus* strain DMKU 3-1042 cultured at 37ºC in sugarcane juice in a medium containing 22% total sugars.

The resulting biomass yield in our study was lower (0.02 g biomass/g sugar) than the typical values (0.03–0.05 g biomass/g sugar) reported for alcoholic fermentation conducted by yeasts [4,21,34]. The low biomass accounted for the low volumetric productivity of ethanol. As pointed out, the lack of an essential nutrient (vitamins, metal ions) might account for the lower yeast growth rate [2]. Another factor may have involved the influence of starvation on the cAMP/PKA pathway, which controls vegetative growth in yeast [20]. These data suggest that larger inocula should be used in order to achieve the necessary process productivity. Ozmihci and Kargi [32] reached similar conclusions in a study of batch ethanol fermentation of cheese-whey powder by *K. marxianus*.

Under optimal conditions with concomitant production of ethanol and endoPG, the enzyme activity in the culture broth was 9.8 U/ml (Table 3), almost 10% higher than the values reported for this same strain in beet molasses [34]. The opti-

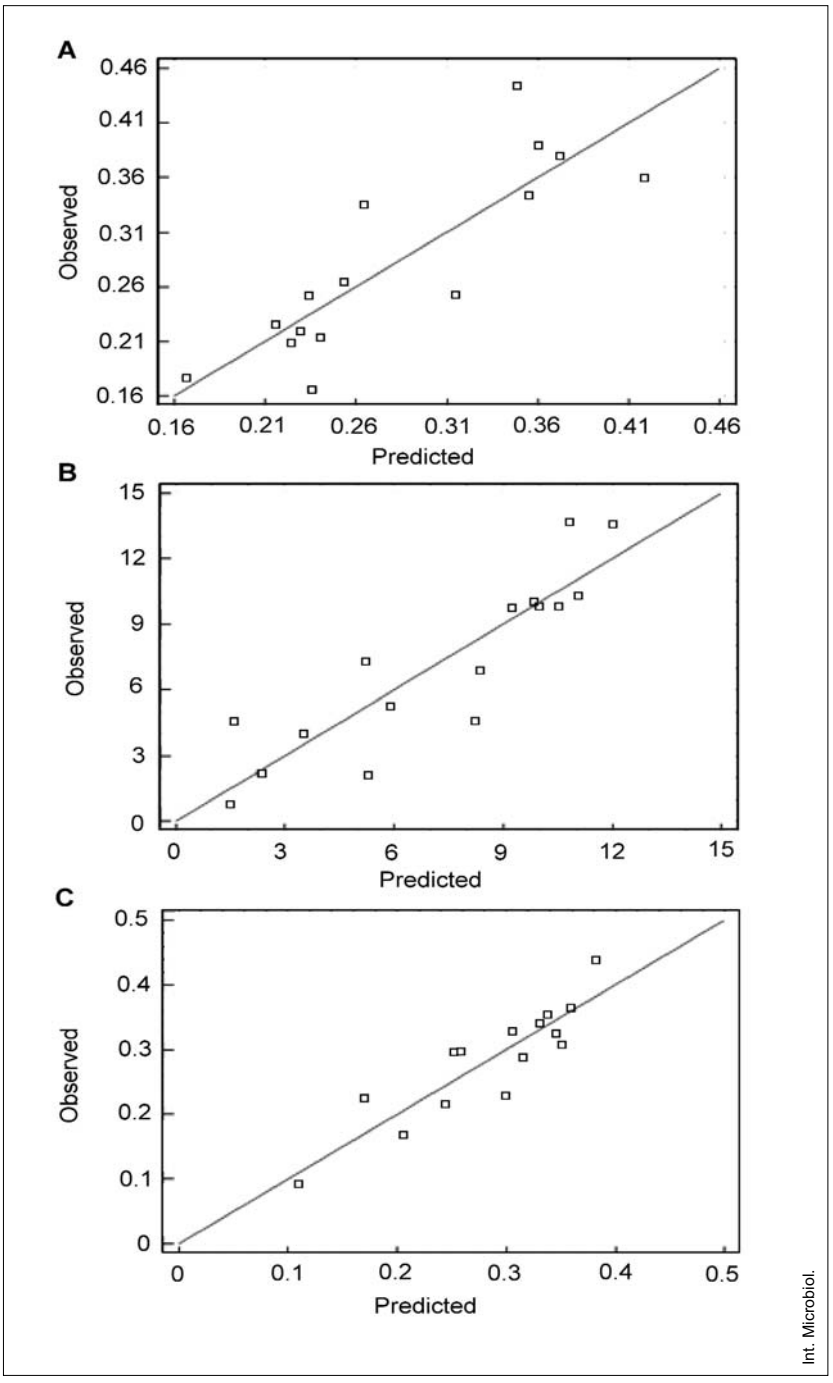

Fig. 2. Parity plots showing the distribution of experimental vs. predicted values of (**A**) ethanol productivity, (**B**) PG activity, and (**C**) integrated response.

mal values of pH (4.6) and temperature (31ºC) determined in the present study are similar to others reported in the literature for PG production by *K. wickerhamii* [28] and *K. marxianus* [10].

Comparison of the results obtained here with those from a previous study [34] suggests that an increase in TRS level (from 100 to 125 g/l), supplementation with nitrogen and nitrogen-phosphorus sources (2.48 g/l $(NH_4)_2SO_4$ and 2.73 g/l $(NH_4)_2HPO_4$), as well as divalent cations Ca^{2+} and Mg^{2+} (0.33 and 0.54 g/l, respectively) would positively affect the co-production of ethanol and endoPG by *K. marxianus* CCEBI 2011.

Influence of environmental and nutritional factors on ethanol and PG production. Once the optimal region where ethanol and PG levels achieve the desired values has been determined, it is important to estab-

Table 4. Coefficients and significance probability of the multiple linear regression models

	Ethanol productivity		PG activity		Integrated response	
Factor	Estimated[a]	*P*-value	Estimated[a]	*P*-value	Estimated[a]	*P*-value
Constant	0.256 ± 0.018	0.000	6.86 ± 0.80	0.000	0.257 ± 0.016	0.000
[H+]	–0.009 ± 0.017	0.628	–0.83 ± 0.77	0.316	–0.024 ± 0.016	0.161
Temperature	0.042 ± 0.017	0.038*	–3.54 ± 0.80	0.003*	–0.048 ± 0.016	0.021*
TRS	0.028 ± 0.017	0.136	0.91 ± 0.75	0.265	0.026 ± 0.015	0.134
$(NH_4)_2SO_4$	0.041 ± 0.015	0.026*	–0.11 ± 0.71	0.877	0.043 ± 0.014	0.019*
$(NH_4)_2HPO_4$	0.023 ± 0.017	0.210	0.62 ± 0.80	0.466	0.024 ± 0.016	0.191
$CaCl_2$	0.020 ± 0.019	0.322	0.56 ± 0.84	0.525	0.027 ± 0.017	0.152
$MgSO_4 \cdot 7H_2O$	–	–	–0.25 ± 0.75	0.752	–0.002 ± 0.015	0.886
	$R^2 = 0.719$		$R^2 = 0.775$		$R^2 = 0.798$	

R^2, coefficient of determination.

[a]Estimated coefficients ± standard error.

*Factors with statistically significant influence ($P < 0.05$).

lish the influence of the different evaluated factors on the responses of interest, either individually or combined (compromise situation). Therefore, a multiple linear regression analysis was carried out that included all experimental trials (Table 4). In all cases, acceptable fits were obtained with $R^2 > 0.7$, and a random behavior of residuals vs. predicted values (data not shown). Parity plots showed a satisfactory correlation between observed and predicted values, and most of the points were found to cluster around the diagonal line (Fig. 2).

Temperature and $(NH)_4SO_4$ supplementation significantly influenced ($P < 0.05$) fermentation, although the latter nutrient was not significant to PG production. The influence of temperature was complex, since higher temperatures increased ethanol productivity but with a concomitant decrease in accumulated PG activity. Temperature, to a certain extent, causes an increase in the yeast growth rate and thus in the ethanol production rate, because ethanol is a by-product associated with energy metabolism. This fact is in agreement with our results in which both the thermotolerance of the strain [33] as well as the maximum temperature (42ºC) were taken into account. The negative effect of temperature on PG production was reported in a previous study of PG synthesis at 30ºC and 40ºC [Serrat M (2003) Ph.D. thesis].

As noted above, satisfactory yeast growth in sugarcane juice required supplementation of the medium with nitrogen and other nutrients. Thus, a favorable influence of $(NH)_4SO_4$ addition on the volumetric productivity of ethanol was to be expected. The non-significant influence ($P > 0.05$) of $(NH)_4HPO_4$ on ethanol production suggests a probable negative effect of the phosphate anion (e.g., on calcium and magnesium availability), which could have masked the positive action of the ammonium ion.

The influence of nutritional factors on PG synthesis in *K. marxianus* is also complex, because production of these enzymes is partially growth-associated, and maximum accumulation occurs as the cultures approach stationary phase [34]. Thus, the establishment of possible correlations with the initial culture composition is difficult and might explain the absence of significant relations between nutritional variables and endoPG production. In addition, the long fermentation times needed, due to the small size of the inocula, could have interfered with the PG values measured, by allowing enzyme degradation or inactivation. This conclusion is consistent with studies carried out on yeast pectinolytic enzymes, in which activity was shown to decrease with increasing culture time [8,18,29].

Note that, in this study, neither TRS concentration nor pH significantly influenced ethanol or PG production. The assayed range of TRS (63–125 g/l) might have masked the occurrence of a possible non-linear effect. In addition, the relatively narrow range of hydrogen ion concentrations (7.1–31.4 μM, equivalent to 0.6 pH units) might have not been enough to allow appreciable changes in the measured responses. In view of the negligible effects of divalent cations (Ca^{2+} and Mg^{2+}) and the nitrogen-phosphorous supplement in the form of $(NH_4)_2HPO_4$, these compounds might be easily suppressed in formulations of sugarcane-juice-based media.

The present work shows that the Simplex method facilitates the establishment—with just a few experiments—of the most favorable fermentation conditions for the simultaneous production of endoPG and ethanol from sugarcane juice by *K. marxianus* CCEBI 2011.

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Competing interests. None declared.

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