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Production, Purification, and Characterization of a Polygalacturonase from a New Strain of *Kluyveromyces marxianus* Isolated from Coffee Wet-Processing Wastewater

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Abstract

A new high polygalacturonase (PG)-producing *Kluyveromyces marxianus* strain was isolated from coffee wet-processing wastewater. PG production in this strain is not repressed in the presence of 100 g/L of glucose and, being growth-associated, reached its maximum accumulation in the culture medium at the beginning of the stationary phase. Oxygen and galacturonic acid negatively regulated enzyme synthesis, and glucose as the carbon source afforded better enzyme yields than lactose. The data reported here show that this strain exhibits the highest index of PG production among the wild-type strains reported so far (18.8 U/mL). PG was readily purified by ion-exchange chromatography on SP-Sepharose FF. The activity corresponded to a single protein with an M_r of 41.7 kDa according to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme was stable in the pH range of 3.0–5.0 and displayed an optimal temperature of 55°C; it showed a typical *endo*-splitting way of substrate hydrolysis and exhibited a fair degree of activity on pectin with a high degree of esterification.

Index Entries: Polygalacturonase; pectin; production; characterization; *Kluyveromyces marxianus*.

Introduction

Pectic substances occur as structural polysaccharides in the primary cell walls and middle lamellae of higher plants and carry out an important

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role in the coherence and integrity of plant tissues *(1,2)*. They constitute a complex heteropolysaccharide, mainly composed of D-galacturonic acid residues joined by α-1,4-linkages and partially methylesterified in what it is known as pectin. Enzymic degradation of pectic substances takes place by the concerted action of pectin methylesterases (PE) and the depolymerizing action of either polygalacturonase (PG) or pectin lyase. PG catalyzes the random hydrolysis of α-1,4-glycosidic linkages between two nonmethylated galacturonic acid residues (endo-splitting PG; EC 3.2.1.15) or the removal of galacturonic acid residues starting from the nonreducing end (exo-splitting PG; EC 3.2.1.67) *(3)*.

Pectic enzymes together with other carbohydrases are widely used in industry, such as in the production of sugar from plant biomass *(4)*; the extraction, clarification, and depectinization of fruit juices (including grape musts); the maceration of fruits and vegetables; and vegetal oil extraction *(5–7)*.

To date, almost all commercial pectinase preparations consist of complex enzymic mixtures of fungal origin, of which, in addition to several pectinolytic activities such as endo- and exo-PG or pectin lyases, certain undesirable pectin methylesterases or arabinofuranosidases and amylases may also be found. In this sense, several investigators *(8)* have suggested the possible advantages of yeast PGs over fungal ones; that is, yeasts such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* produce only endo-PG and do not show PE activity.

K. marxianus is one of the few ascomycetous yeasts reported to be able to degrade pectin and, in fact, is thought to play an important role in the breakdown of the pectinacious layer surrounding coffee and cocoa beans during processing *(9,10)*. Schwan et al. *(11)* have characterized the PG of an *K. marxianus* strain isolated from cocoa pulp fermentation, while Jones and Jones *(12)* have described the participation of several yeasts species in coffee mucilage solubilization.

Here we report on the isolation of a new high polygalacturonase-producing strain of *K. marxianus* from coffee fermentation wastewaters, as well as on a study of the influence of culture conditions on PG production in this strain. Finally, we describe the purification and biochemical characterization of the enzyme.

Materials and Methods

Isolation of Yeast

Samples of wet coffee-processing wastewaters from Filé, Santiago de Cuba, Cuba, were left to ferment spontaneously for 24 h. They were then inoculated (5% [v/v]) into 100-mL flasks containing 20 mL of malt extract broth (pH 3,5; YME, Oxoid) and incubated under conditions of shaking (200 strokes/min) at 30°C for 48 h. Appropriate dilutions were plated onto YME agar (pH 3.5) to obtain isolated yeast colonies. Clones were tested on plates for pectinolytic activity, and those found to be positive were identi-

fied and classified according to the criteria of Barnet *(13)*. The isolate with highest pectinolytic activity was found to be *K. marxianus* and was named CCEBI 2011 (Culture Collection of the Studies Center of Industrial Biotechnology, Universidad de Oriente, Santiago de Cuba, Cuba). Throughout this work, the strain was maintained on YME slopes at 4°C.

Medium and Inoculum

Unless otherwise stated, liquid medium containing 6.7 g/L of YNB (Difco) and 10 g/L of glucose (pH adjusted to 5.4) was used in all experiments. The inoculum consisted of 1% (v/v) 12-h-old YPD culture (5×10^6 cells/mL) in all cases.

Assay of Pectinolytic Activity in Plates

Pectinolytic activity was detected on plates containing 6.7 g/L of YNB, 5 g/L of glucose, 5 g/L of polygalacturonic acid (Sigma), and 20 g/L of bacteriologic agar (Difco), with the pH adjusted to 5.4. Plates were incubated at 30°C. After 72 h, enzyme activity was visualized by hydrolysis halos after the plates had been flooded with 6 *M* HCl *(14)*.

Influence of Culture Conditions on PG Production

Oxygen Concentration

Three variations in the rotational speed (0, 100, and 200 min^{-1}) were used to assess three different amounts of dissolved oxygen (DO) in the culture media. Cultures were made up in 100-mL flasks containing 20 mL of liquid medium. Flasks were inoculated and incubated at 30°C for 12 h. PG activity, reducing sugars, and biomass were then measured. Three replicas were used for each condition.

Carbon Source

Experiments were carried out in culture tubes (100 × 14 mm) containing 4 mL of liquid medium. The following carbon sources were used: glucose, galacturonic acid, and polygalacturonic acid plus lactose, all prepared at 10 g/L except galacturonic or polygalacturonic acids, which were at 5 g/L, and glucose, which was occasionally at 100 g/L. Tubes (three replicas) were incubated at 30°C and tested every 24 h over 3 d for biomass, PG activity, and protein production.

Enzyme Production and Purification

A 500-mL flask containing 400 mL of medium was inoculated and incubated without shaking at 30°C. After 24 h, the culture was centrifuged at 10,000*g* for 20 min, and 250 mL of the supernatant was filtered through a 0.45-µm filter (Millipore) and concentrated by ultrafiltration in Filtron Omega devices (number molecular weight limit [NMWL] 10 kDa) down to 25 mL. The concentrate was then dialyzed against 50 m*M* sodium acetate buffer, pH 4.5, and loaded onto an SP-Sepharose Fast Flow (Pharmacia

Biotech) cation-exchange chromatography column (16 × 1 cm), previously equilibrated with the same buffer. All PG activity was retained in matrix and was successfully eluted at a flow rate of 0.4 mL/min with a linear gradient of NaCl (0–150 m*M*) prepared in 50 m*M* sodium acetate buffer, pH 5.0. Fractions containing PG activity were pooled for further studies.

Enzymic Assays

Prior to all enzymic assays, protein in the cell-free samples was precipitated with chilled acetone (75% [v/v]) and centrifuged for 5 min at 14,000*g*. The pellet was then washed twice in chilled absolute ethanol and pelleted. The protein was finally redissolved in 50 m*M* sodium acetate buffer, pH 5.0. Pectin methylesterase and pectin lyase activities were determined by the methods of Albersheim *(15)* and Alaña et al. *(16)*, respectively.

PG activity was estimated from the increase in reducing power from 1% (w/v) polygalacturonic acid in 50 m*M* sodium acetate buffer, pH 5.0, at 37°C for 10 min and was evaluated by Somogyi's *(17)* method as modified by Nelson *(18)*. A typical reaction mixture contained 400 µL of substrate and 100 µL of the appropriate enzyme dilution in 50 m*M* sodium acetate buffer, pH 5.0. One unit of PG activity was defined as the amount of enzyme producing 1 µmol/min of galacturonic acid or equivalent reducing power under these conditions.

Polygalacturonic acid (sodium salt; Sigma, St. Louis, MO) and pectin (from apples, M_r = 30,000–100,000; degree of esterification = 70–75%; Fluka) at 0.5% (w/v) in 50 m*M* acetate buffer, pH 5.0, were used as substrates when residual viscosity was to be evaluated. The reaction mixtures contained 4.9 mL of substrates and 0.1 mL of enzyme preincubated at 37°C. Viscosity was then measured at zero or appropriate times using a Canon-Fenske capillary viscosimeter (model 5354/2). The degree of hydrolysis was estimated on the basis of the galacturonic acid content of the different substrates.

Analytical Electrophoresis

Determinations of the purity and molecular weight of PG were performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a slab gel prepared with 12% (resolving gel) and 5% (stacking gel) polyacrylamide according to Ausubel et al. *(19)*. After electrophoresis, the gels were subjected to Coomassie blue G-250 staining.

Other Analytical Determinations

Protein was determinate by the Folin phenol reagent, using bovine serum albumin as the standard *(20)*. Biomass was measured spectrophotometrically at 620 nm using a standardized gravimetric curve and, when necessary, galacturonic acid was assayed by the carbazol colorimetric method *(21)*. Ethanol was determined using a Boehringer Mannheim kit, carrying out the reactions as recommended by the manufacturer.

Results and Discussion

Isolation of Pectinolytic Yeast Strain

During wet coffee processing, depulped coffee beans are placed under natural fermentation to remove the mucilaginous cover, which is mainly formed by pectin. This pectinaceous layer is partially degraded by microbial action and later removed through successive washings in water. Naturally, this fermentation is carried out by yeasts; the wastewaters were used as the starting material for the present work on the isolation of pectinolytic yeasts and the characterization of the enzymic system.

To favor the development of the native microbiota, a sample of the wastewater was allowed to ferment spontaneously for 24 h. Then, an aliquot (1 mL) was inoculated into 20 mL of YEM medium (pH 3.5) to promote yeast growth. Finally, the appropriate dilutions were plated onto the same medium, and individual yeast colonies were obtained. On the basis of colony observation, 30 strains were chosen to evaluate their in-plate pectinolytic activity, the result being a single isolate exhibiting pectinolytic activity. The isolate was classified in clear agreement with Barnet's keys for yeast identification *(13)* as *K. marxianus*. This yeast species has been reported to be one of the best pectinolytic enzyme producers that leads to natural cocoa fermentations *(11)*. The strain was deposited at the Culture Collection of the Studies Center of Industrial Biotechnology, Universidad de Oriente, Santiago de Cuba, Cuba, under the denomination of *K. marxianus* CCEBI 2011.

Influence of Culture Conditions on PG Synthesis

Previous studies have demonstrated that PG production in yeasts tends to be constitutive and is repressed by high glucose concentrations and by oxygen *(22)*. Regarding the effect of galacturonic or polygalacturonic acids, contradictory results have been described. For example, Blanco et al. *(22)* have reported that in *S. cerevisiae* polygalacturonic acid respectively stimulates and represses PG synthesis, whereas Schwan and Rose *(23)* claim that pectin or polygalacturonic acid has no influence on PG production in *K. marxianus*. Also conflictive are the results reported for *S. cerevisiae* and *K. marxianus* regarding the effect of different carbon sources on PG synthesis. Reports on the constitutive nature of yeast PG synthesis have frequently been based on the observation of hydrolysis halos in plates containing polygalacturonic acid plus glucose. In the present study, the standard inoculum used consisted of 1 µL of cell suspension (5×10^5 twice-washed cells) in sterile distilled water. It was found that regardless of the carbon source employed, hydrolysis halos were always present, although they were greater for 5 g/L of glucose; given that pectinolytic activity is directly proportional to the areas, pectinolytic activity with 100 g/L of glucose would represent about 75.8% of that of 5 g/L.

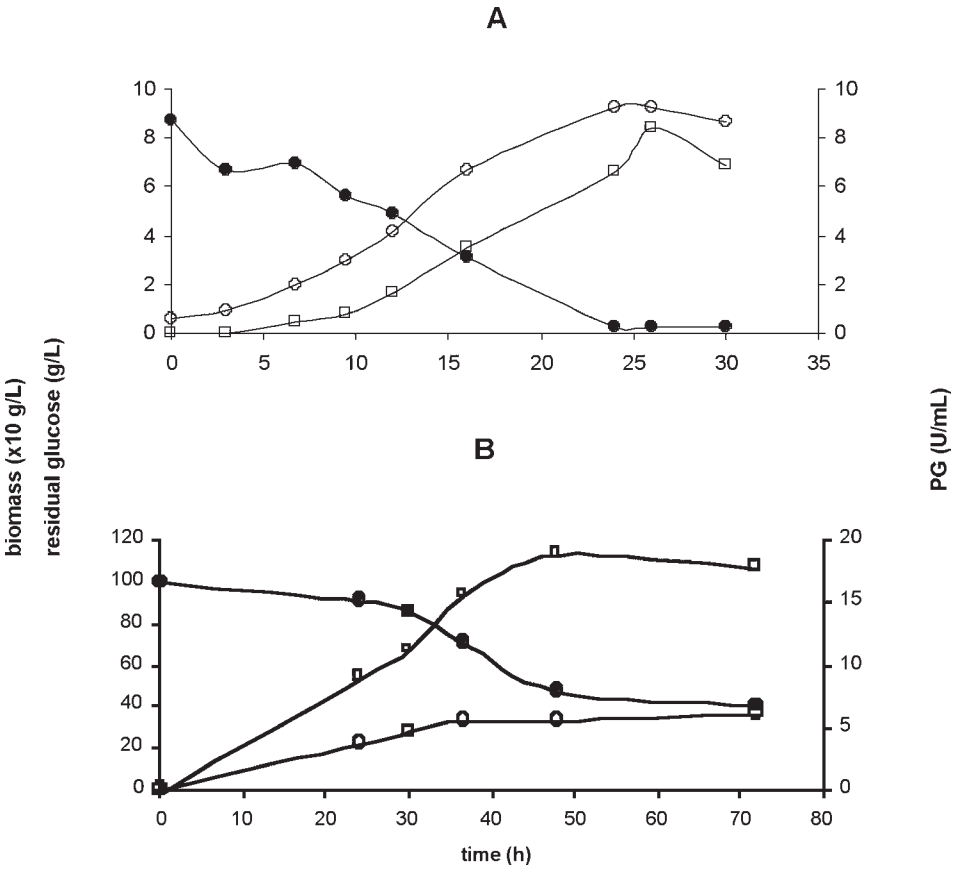

Fig. 1. Kinetics of PG production in YNB-glucose medium by *K. marxianus*. **(A)** 10 g/L of glucose; **(B)** 100 g/L of glucose. ○, Biomass; ●, residual glucose; □, PG activity.

These results corroborate the constitutive nature of PG in yeasts even in the presence of high glucose concentrations. Thus, PG synthesis in this strain constitutes a singular example of an expression system that is not repressed by catabolites. This is crucial if this strain is to be used for the simultaneous production of PG and ethanol, starting from high-glucose raw materials.

The results concerning the growth and PG production in *K. marxianus* grown under nonshaking conditions are shown in Fig. 1; neither pectin methylesterase nor pectate lyase activities were detected in the culture filtrates. As can be seen, PG production was associated with microbial growth, largely independent of the glucose concentrations, and increased steadily until the stationary phase was reached. These results confirm those previously reported by Schwan and Rose *(23)*. In the case of 100 g/L of glucose, after 48 h of culture, when the glucose concentration had fallen to almost half the initial value, a considerable decrease in the growth rate was

observed (Fig. 1B), possibly owing to the accumulation of ethanol. This would be in agreement with the relatively low ethanol tolerance reported for *K. marxianus* strains *(24)*.

The influence of DO on PG production was studied for three oxygenation conditions; the results are shown in Table 1. The biomass yield as a function of the substrate consumed ($Y_{X/S}$) increased as did the degree of oxygenation, whereas PG production decreased fourfold. Thus, enzyme yields as a function of biomass units were 3–11 times higher in static cultures than in highly oxygenated ones. In this case, complete repression of pectinolytic activity was not observed, even at the highest rotational speed, unlike the findings reported by Blanco et al. *(22)* for *S. cerevisiae*. Maximal PG production occurred when the cultures approached the stationary phase, the glucose had fallen to below 1 g/L, and the culture had become oxygen limited by excess biomass. In light of this experimental evidence, it is also possible that PG genes in yeasts could be regulated by stress factors other than anaerobiosis, such as specific nutrient limitation or osmotic stress caused by the pectin present in the econiches of pectinolytic yeasts. Thus, other proteins, such as Hsp 150p *(25)* and Ygp1p, are secreted into the medium in response to nutrient limitation or the presence of ethanol *(26,27)*.

Table 2 gives the results on the influence of the carbon source on growth and PG production. In the presence of either galacturonic or polygalacturonic acids as the sole carbon source, no growth was observed, as also reported by Schwan et al. *(11)*. The growth rate and biomass yield were supported by lactose but to a lower extent compared with glucose. Finally, the mixture of 10 g/L of glucose plus 5 g/L of galacturonic acid afforded a slightly lower biomass yield compared with the medium containing 10 g/L of glucose alone.

The highest extracellular PG accumulation (18.8 U/mL) was obtained with 100 g/L of glucose in a 48-h culture; to the best of our knowledge, this value is the highest yet reported for a wild-type yeast. Enzyme yield per unit of biomass was also the highest, and this is consistent with the hypothesis that PG production is associated with cells that display fermentative metabolism.

The addition of pectic substances (galacturonic and polygalacturonic acids) diminished PG production; this effect was more pronounced for the monomer. Schwan and Rose *(23)* found that additions of pectin and polygalacturonic acid up to 1% did not affect PG synthesis in *K. marxianus*. However, Blanco et al. *(22)* observed that 0.2% polygalacturonic acid inhibited PG synthesis in *S. cerevisiae* and that the addition of galacturonic acid (0.5%) increased PG production considerably. These results are in agreement with biomass yields observed. Thus, it can be evoked until Northern-type results are obtained that pectic substances affect the general metabolism of the cells under the experimental conditions evaluated here, with this effect more evident for the monomer.

Table 1
Growth, PG Production, Biomass/Substrate and Enzyme/Biomass Yields Under Different Oxygenation Conditions

Oxygenation (as rotation, in min^{-1})	Biomass (mg/mL)	Biomass/substrate yield	PG activity (U/mL)	Enzyme/biomass yield (U/mg)
0	1.3	0.16	2.4	1.8
100	2.7	0.27	1.6	0.6
200	4.1	0.41	0.6	0.15

Table 2
Influence of Nature and Concentration of Carbon Source on Growth and PG Production[a]

Carbon source	Biomass (mg/mL)	PG activity (U/mL)	Enzyme/biomass yield (U/mg)	Enzyme productivity (U/[mL·h])
Glucose (10 g/L)	1.1	10.2	9.3	0.43
Glucose (10 g/L + 5 g/L galacturonic acid)	0.8	5.5	6.9	0.18
Glucose (10 g/L + 5 g/L polygalacturonic acid)	1.0	7.4	7.4	0.31
Lactose (10 g/L)	0.8	2.2	2.7	0.04
Glucose (100 g/L) (24 h)	1.2	9.0	7.5	0.37
Glucose (100 g/L) (48 h)	1.7	18.8	11.1	0.39

[a]All measurements correspond to cultures that had just reached the stationary phase of growth, except for 100 g/L of glucose variant; three replicas for each variant.

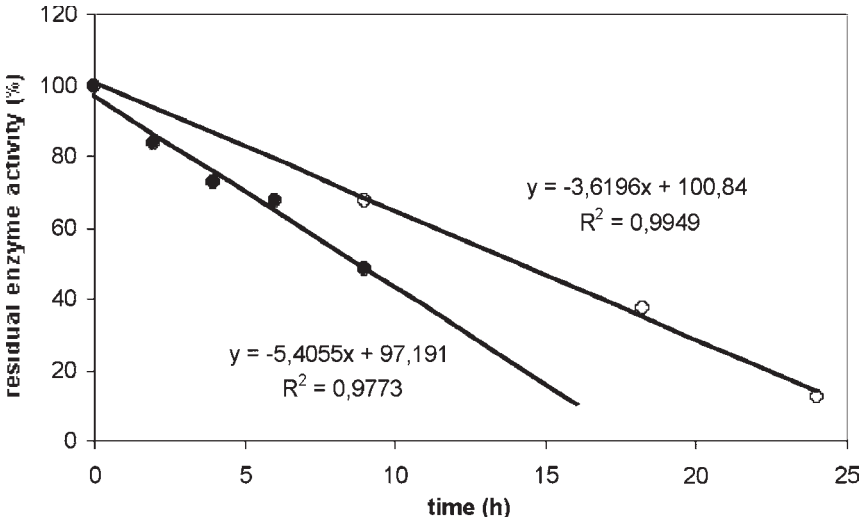

Fig. 2. Thermal inactivation of *K. marxianus* PG. ○, 50°C; ●, 55°C.

Characterization and Purification of Enzyme

The enzyme was active in the pH range of 3.0–5.0, with the optimum at 4.5. At pH 3.0, the enzyme showed 50% maximum activity and was completely inactivated at pHs above 6.0, similar to other yeast PGs *(22,28,29)*. The still high residual activity of the *K. marxianus* PG at pH 3.0 is interesting in terms of possible application in the fruit juice–processing industry, where acidic conditions are usual.

Enzyme activity increased steadily between 20 and 55°C, falling off sharply beyond this point. The temperature of 55°C is significantly higher than the 40 or 45°C reported by Schwan et al. *(11)* and Blanco et al. *(22)* for the PGs of *K. marxianus* and *S. cerevisiae*, respectively. The enzyme was relatively stable at 50 and 55°C, with 50% of the activity persisting after 14 and 8.7 h, respectively. At 60°C, the enzyme was completely inactivated in 6 min. As shown in Fig. 2, PG thermal denaturation displayed first-order kinetics, which allowed us to calculate, using the Arrhenius plot, an activation energy for its thermal denaturation of 498.5 kJ/mol, showing that the *K. marxianus* PG is moderately thermostable. The values of thermal stability and optimal pH found here are similar to those reported for a *Pichia pinus* strain growing on mango wastes *(30)*.

The supernatant containing PG activity was concentrated 10-fold (as specified in Materials and Methods), dialyzed overnight against 50 m*M* sodium acetate buffer (pH 4.5), and applied to an SP-Sepharose FF column previously equilibrated with the same buffer; the enzyme was retained at that ionic strength. When a 0–150 m*M* NaCl gradient was applied, PG activity eluted as a single peak (Fig. 3). SDS-PAGE of this peak revealed a single band with an apparent M_r of 41.7 kDa (Fig. 4). The results of the sample purification steps are summarized in Table 3; PG represented about

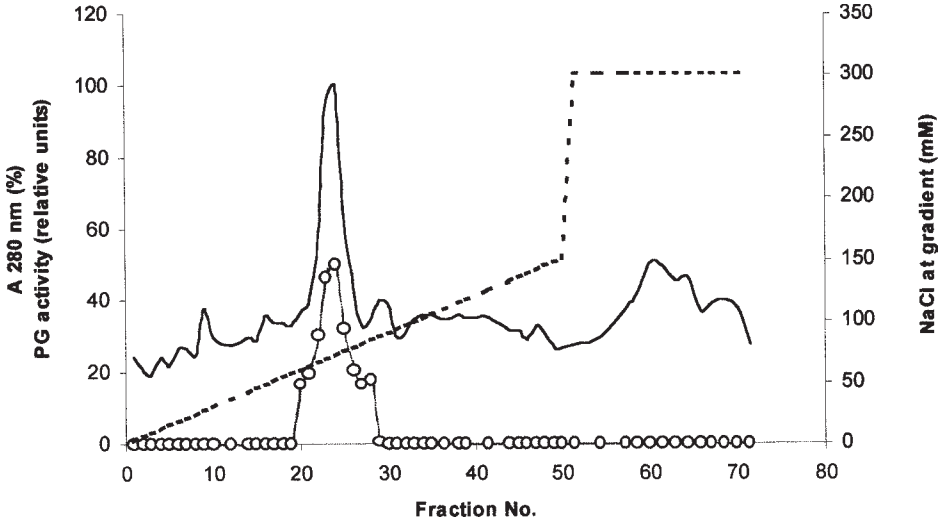

Fig. 3. Protein elution profile in SP-Sepharose column under an NaCl gradient in 50 m*M* sodium acetate buffer (pH 5.0). (——), Absorbance at 280 nm; (—○—), PG activity; (– – –), NaCl gradient.

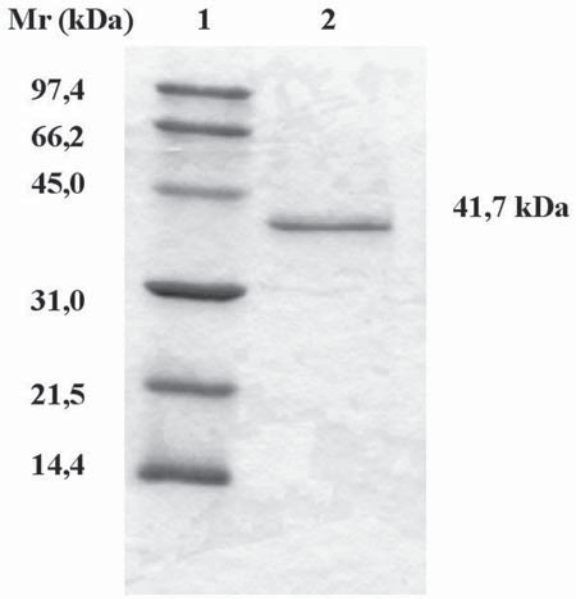

Fig. 4. SDS-PAGE of PG active fraction from ion-exchange chromatography. Lane 1, low molecular weight standards; lane 2, PG.

75–80% of all the protein secreted by *K. marxianus* into the medium, accounting for the slight increase in specific activity (9.2-fold) observed during purification. The apparent M_r is somewhat different from the values

Table 3
Summary of PG Purification from Culture Fluid of *K. marxianus*

Purification step	Volume (mL)	Total proteins (μg/mL)	Enzyme activity (U/mL)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
Culture fluid	250	121	2.9	725.0	24	1	100
Concentration	24	139	22.3	535.2	160	6.7	74
SP-Sepharose chromatography	5.5	256	56.0	308.0	219	9.1	42

Table 4
Kinetic Constants for PG of *K. marxianus* on PGA and Highly Esterified Pectin

Substrate	k_{cat} (S^{-1})	K_M (mg/mL)	K_M (mM)[a]	k_{cat}/K_M ($S^{-1}\cdot M^{-1}$)
PGA	1.83	0.03	0.17	1.1×10^4
Apple pectin (75% d.e.)[b]	6.08	8.6	48.8	124.2

[a]As anhydrogalacturonic acid.
[b]d.e., degree of esterification.

described by Schwan et al. *(11)*, who reported four bands of M_r of 45, 42, 39, and 36 kDa, respectively, all exhibiting PG activity. However, according to them the bands could represent the same enzyme with different degrees of glycosylation. According to Šiekštelė et al. *(31)*, unglycosylated or underglycosylated PG would consist of a polypeptide of 34.7 kDa, this protein displaying two possible glycosylation sites. Thus, good correspondence is seen between the purified PG and the reported sequence of the PG gene of *K. marxianus*.

For the purified enzyme, the kinetic constants for polygalacturonic acid and highly esterified pectin were determined with purified enzyme (Table 4). k_{cat} and K_M were found to be three times and two orders higher, respectively, for the esterified substrate than for polygalacturonic acid (PGA), leading the catalytic efficiency (k_{cat}/K_M) to be considerably higher for the nonesterified substrate. This is in agreement with results published for other yeast PGs *(32)* and confirms that PGs are enzymes that catalyze the hydrolysis of α-1,4-glycosidic bonds between nonesterified galacturonic acid residues. The K_M value described here for polygalacturonic acid is the lowest yet reported for yeast PGs.

Figure 5 shows the behavior of *K. marxianus* PG on both PGA and highly esterified pectin. As may be seen, the enzymic reaction on the nonesterified substrate followed a linear trend, even with 12% of the glycosidic bonds already hydrolyzed (Fig. 5B), thus confirming reports by Demain and Phaff *(33,34)* in their pioneer studies with the PG of *S. cerevisiae*. However, when pectin was used as the substrate, the results were different; linearity was lost when as few as 2% of the linkages were hydrolyzed (Fig. 5B). Natural pectins are randomly esterified but contain blocks of nonesterified regions *(35)*. These blocks must be the target for yeast PGs, but when they are reduced to less than five units of galacturonic acid, the overall reaction slows down *(33,34)*. Figure 5A relates the viscosity to the percentage of hydrolyzed bonds. As can be observed, viscosity was reduced to 50% when only as few as 1 and 3% of the glycosidic linkages were hydrolyzed in pectin and polygalacturonic acid, respectively. This result indicated that we were dealing with a typical *endo* PG, with a random mode of action on the polygalacturonate chain, which is a common situation in yeast PGs (for a review, see Blanco et al. *[8]*).

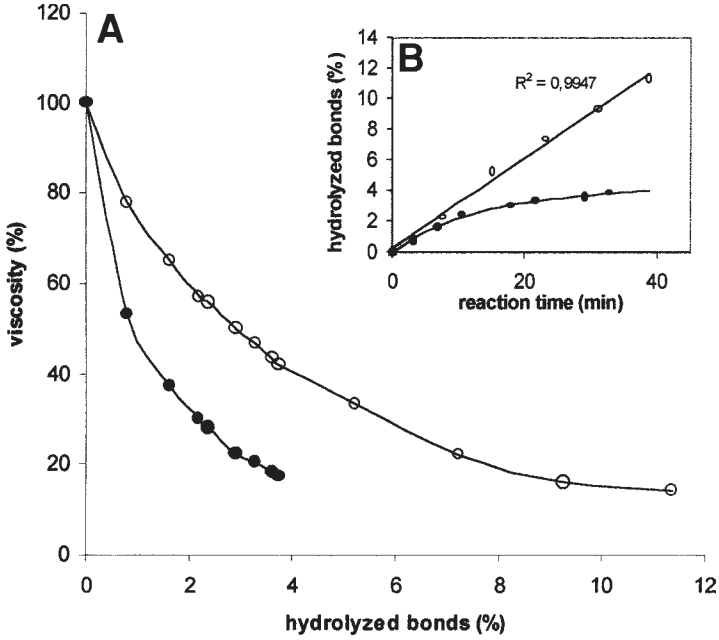

Fig. 5. Hydrolytic behavior of *K. marxianus* PG on PGA (○) and pectin (●). **(A)** Viscosity decrease with time; **(B)** enzyme activity vs reaction time.

Finally, the enzyme showed a high macerant capacity on highly esterified pectin and was in fact able to diminish its viscosity to 17% in 2 min at 37°C when the enzyme activity in the reaction mixture was as low as 1 U/mL (data not shown).

Conclusion

The study reported here on the wild-type PG-producing strain *K. marxianus* CCEBI 2011, isolated from wet coffee-processing wastewater in Cuba, contributes to extending our knowledge about the factors affecting PG production in yeasts, such as negative regulation by oxygen and galacturonic acid. Although much remains to be investigated concerning the biologic function/actual role of PGs in yeasts and the mechanisms of their regulation at genetic and molecular levels, the hypothesis that this enzyme would be produced as a cellular response to adverse environmental conditions *(36)* is somewhat reinforced by the results obtained. Regarding the carbon source, our results demonstrate that this is a crucial parameter to be taken into account for optimizing PG production. Finally, the high levels of PG production in this particular strain, as well as the PG synthesis observed to occur in the presence of high levels of glucose, its reasonable thermal stability, and macerant capacity on highly esterified pectin of the enzyme, are relevant features for considering this strain as a good candidate for industrial exploitation.

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