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Polygalacturonase and Ethanol Production in *Kluyveromyces marxianus*

Potential Use of Polygalacturonase in Foodstuffs

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Abstract

The coproduction of ethanol and polygalacturonase (PG) in a pilot-scale batch fermentor using yeast extract—glucose (YD)—and sugar beet molasses (SBM)-based media was implemented utilizing a new high-PG-producing strain of *Kluyveromyces marxianus*. A certain growth inhibition was observed in SBM medium, causing ethanol and PG production to be lower. Ethanol productivity and accumulation values of 1.94 g/(L·h) and 40 g/L, respectively, were attained in YD, whereas the best fermentation efficiency (95.1%) was achieved with SBM medium. Maximal PG synthesis occurred at the end of cell growth, with values of 1.08 and 0.46 U/(mg·h) for the YD and SBM media, respectively. When the cultures reached stationary phase, PG production stopped. The highest accumulation level (17 U/mL) occurred in YD medium, in agreement with previous laboratory-scale studies carried out for this strain. The potential applications of the crude enzyme preparations were evaluated with different fruit juices and vegetable slices. The enzyme was able to increase the filtration rate of orange, pear, and apple juices by twofold. Additionally, complete clarification of apple juice was readily accomplished, whereas cucumber, carrot, and banana tissues were macerated to a lesser extent.

Index Entries: Yeast; *Kluyveromyces marxianus*; polygalacturonases; food processing; ethanol; beet molasses.

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Introduction

Pectic substances are a main component of the cell walls of higher plants and consist of a backbone of α-1,4-D-galacturonic acid residues that are partially esterified. The enzymes that hydrolyze pectic substances are known as pectic enzymes, pectinases, or pectinolytic enzymes. Pectinases are divided into pectinesterases and depolymerizing enzymes. The latter group includes polygalacturonases (PGs), which operate through a hydrolytic mechanism, and lyases, which act by transeliminative cleavage *(1)*.

Pectinolytic enzymes are of considerable commercial importance *(2)* in the sense that they are used in many industrial applications, mainly in fruit and vegetable processing. Such applications include the extraction and clarification of fruit juices, the maceration of vegetables, wine making, oil extraction *(1,3–5)*, the production of fermentable sugars from plant biomass *(6)*, the retting of textile fibers *(7)*, coffee and cocoa curing *(8,9)*, and functional foodstuff elaboration *(10)*.

Commercial pectinases are produced from *Aspergillus niger*, and the preparations involved are a complex mixture of several pectic and nonpectic enzymes, including cellulases, amylases, and arabinofuranosidases. Generally, the presence of some of these enzymes is undesirable because the quality parameters of the enzymatically treated product may be affected (e.g., methanol production, browning). Accordingly, the search for new sources of pectic enzymes is currently a "hot" field of research. In this sense, yeasts provide an alternative source of pectinases *(11,12)*.

In a previous work, we described aspects concerning the production, physiology, and biochemical characterization of the PG from a new high-producing strain of *Kluyveromyces marxianus* isolated from the wastewater from wet processing of coffee *(13)*. In this article, we report a study of PG and ethanol coproduction in a batch process at pilot scale. We also examine the enzyme's applications in food processing.

Materials and Methods

Preparation of Microorganisms and Inoculum

K. marxianus CCEBI 2011 (Culture Collection of the Industrial Biotechnology Studies Centre, Universidad de Oriente, Santiago de Cuba, Cuba) was stored on malt extract agar slants at 4°C. For inoculum preparation, a 250-mL conical flask containing 50 mL of yeast maintenance medium (YMM) consisting of 3 g/L of yeast extract, 5 g/L of peptone, 3 g/L of malt extract, and 10 g/L of glucose, pH 5.0, was inoculated with a single loopful of the microorganisms from the malt extract agar slants. The flask was then incubated at 30°C in a rotary shaker at 200 min^{-1} for 12 h. Next, the growing cultures were inoculated (2% [v/v]) into two 5-L conical flasks containing 1 L of YMM and incubated for an additional 12 h under the same conditions. These cultures were then used as inoculum for the 100-L fermentor.

Molasses

Sugar beet molasses (SBM) was supplied by Piensos Compuestos Día, León, Spain. The content of the reducing sugars was 49.5% (w/v) and the density was 1.396 g/mL.

Pilot-Scale Batch Fermentation

Batch cultivation was carried out at 30°C, pH 5.0, in a Biostat D® fermentor (B. Braun Biotech, Melsungen, Germany) with a 100-L working volume. Before adjusting the pH, YD (10 g/L of yeast extract, 100 g/L of glucose, pH 5.0) or SBM (for 100 g/L of reducing sugars; 1.3 g/L of ammonium sulfate, pH 5.0) medium was sterilized *in situ* at 110°C for 20 min. The pH was maintained at 5.0 by automatic addition of 2*N* KOH or 10% H_2SO_4, and the stirring speed was kept at 5*g*. Air was supplied at 15 L/min (0.15 vvm) over the first 4 h of fermentation. The pressure in the fermentor was adjusted manually to 0.5 atm. At different fermentation intervals, 100-mL aliquots of cultured samples were collected and centrifuged for biomass separation; cell-free broths were stored at –20°C until analytical determination. Fermentation was stopped at the early stationary phase. Then, the culture was centrifuged in a Beckman J2-MC centrifuge, using the JCF-Z continuous operation rotor. Centrifugation was accomplished at 8000*g*, 10°C with a feed flow of 250 mL/min. The enzyme present in the cell-free supernatant was concentrated fivefold using an industrial membrane concentrator (cutoff limit of 30 kDa; Seta, Madrid, Spain) according to the manufacturer's guidelines, aliquoted into 2-L plastic flasks, and stored at –20°C. This enzymic crude was used in application assays. The fermentation was carried out three times.

Calculation Procedures

Specific growth rate (μ) was calculated as follows:

$$\mu = \ln(X_2/X_1)/(t_2 - t_1) \quad (1)$$

in which X_2 and X_1 are biomass concentrations at the beginning and end of the time interval $(t_2 - t_1)$. The μ value calculated in this way is matched with the average ethanol concentration ($\overline{E}_{1,2}$) for this time interval, which is calculated as follows:

$$\overline{E}_{1,2} = (E_1 + E_2)/2 \quad (2)$$

Volumetric (RP_v) and specific (RP_{sp}) enzyme production rates were calculated as follows:

$$RP_v = (P_{v2} - P_{v1})/(t_2 - t_1) \quad (3)$$

$$RP_{sp} = (P_{sp2} - P_{sp1})/(t_2 - t_1) \quad (4)$$

in which $P_{v2,1}$ are the volumetric enzyme production at two times (t_1 and t_2) (U/mL) and $P_{sp2,1}$ are the specific enzyme production at the same times

(U/mg of biomass). P_{sp} for a given time t_i is calculated through the following expression:

$$P_{spi} = P_{vi}/X_i \quad (5)$$

in which X_i is the biomass concentration at that time.

Volumetric and specific enzyme production rates are matched to the average time for the interval. The time intervals considered for the calculation of all these parameters were usually of 1 h.

Enzyme Assay

Cell samples were 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 centrifuged again. The protein was finally redissolved in 50 m*M* sodium acetate buffer, pH 5.0. PG activity was estimated from the increase in reducing power from 0.5% polygalacturonic acid in 50 m*M* sodium acetate buffer, pH 5.0, at 37°C for 10 min and was evaluated using the method of Somogyi *(14)* as modified by Nelson *(15)*. The 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 enzyme activity was defined as the amount of enzyme producing 1 µmol/min of galacturonic acid or equivalent reducing power under these conditions. The stability of the enzymatic crude was assayed at several pHs, at which the enzyme was incubated over 24 h at 4°C in 10 m*M* sodium citrate buffer (pH 3.0–6.0) or sodium phosphate buffer (pH 6.0) *(5–7)*, and then the enzyme activity was estimated according to the procedure just described.

Analysis

Biomass was estimated as cell dry mass after three washes with demineralized water and drying in a vacuum oven at 80°C for 12 h. Reducing sugars in the medium were determined according to the method of Somogyi-Nelson. Acid hydrolysis by Walker's method was carried out before determination of total reducing sugars in SBM medium *(13)*. Ethanol was determined using a Boehringer Mannheim kit, carrying out the reactions as recommended by the manufacturer. Total and volatile fatty acids, luminance, and color were determined according to APHA standard methods *(16)*. Protein was estimated by the Folin phenol reagent, using bovine serum albumin as standard *(17)*.

Application Assays

Ripe and healthy fruits of Valencia Late orange (*Citrus sinensis* L.), Belgian Conference pear (*Pyrus communis* L.), and Red Delicious apple (*Malus domestica* Borkh.) were used to obtain the corresponding juices or pulps, which were prepared with a domestic juice extractor and blender, respectively. Then, the enzyme was added (1 U/mL in juices and 1 U/g in pulps) and the samples were incubated at 45°C for 60 and 120 min.

Next, solids were removed by centrifugation (5000*g*, 15°C, 20 min) and weighed (wet weight). The gross and low-density solids present in orange juice were removed by filtration through a stainless steel strainer (0.5-mm inner-pore diameter) before centrifuging. The absorbance at 650 nm of the free solid supernatants was measured, and the degree of clarification was estimated from the reduction of these absorbance values. Supernatants (250 mL) were vacuum filtered (55 mmHg) through Whatman No. 1 paper (4.7 cm diameter) (Whatman, Kent, England), and the filtration time was measured. Firm and green fruits of cucumber (*Cucumis sativus* L.); healthy tubers of carrot (*Daucus carota* L. ssp. s*ativa*); and firm, ripe fruits of Burro CENSA (Havana, Cuba) banana (*Musa paradisiaca* L.) were used to assay the soaking activity of yeast PG on vegetal tissues. The experimental procedure was carried out according to the method developed by Mussell and Morre *(18)* for PG bioassay. Assays were performed at 37°C, pH 5.0 (50 m*M* sodium acetate buffer), 1 U/mL of PG activity over 120 min. Soaking activity is expressed as the percentage of loss of weight in the tissue subjected to the enzymatic treatment. A control with thermally inactivated enzyme and five replicas was also performed in all assays.

Statistical Analyses

Significant differences between the results were calculated by one-way analysis of variance. Differences at $p < 0.05$ were considered to be significant. All statistical analyses were performed using the Statgraphics Plus 2.1 (Statistical Graphics, Rockville, MD) program.

Results

Ethanol and PG Production in Batch Processes

Figure 1 shows the kinetics of fermentation, and the physiologic and yield parameters are summarized in Table 1. In SBM medium, the maximum specific growth rate was half that obtained for YD medium (0.562 h^{-1}), and, as a consequence, the fermentation time was twofold longer, whereas the biomass yield in SBM was almost half that reached in YD. Additionally, an exponential drop (regression value: 0.98) in specific microbial growth rate was observed for ethanol concentrations higher than 3 g/L (Fig. 2), also in agreement with the switch to anaerobic metabolism.

Ethanol was accumulated at an approximately constant rate during the course of fermentation (Fig. 3), with volumetric yields of 1.94 g/(L·h) (YD) and 1.37 g/(L·h) (SBM). The specific ethanol production rate underwent a striking decrease when accumulated ethanol was above 3 g/L (Fig. 2) and was even lower for concentrations higher than 10 g/L. When the ethanol concentration reached 25 g/L, microbial growth almost halted, although ethanol production continued until total substrate depletion (Figs. 1 and 2). The ethanol accumulated in YD and SBM media at the end of the fermentations (about 40 g/L) (Table 1) was similar and matched the

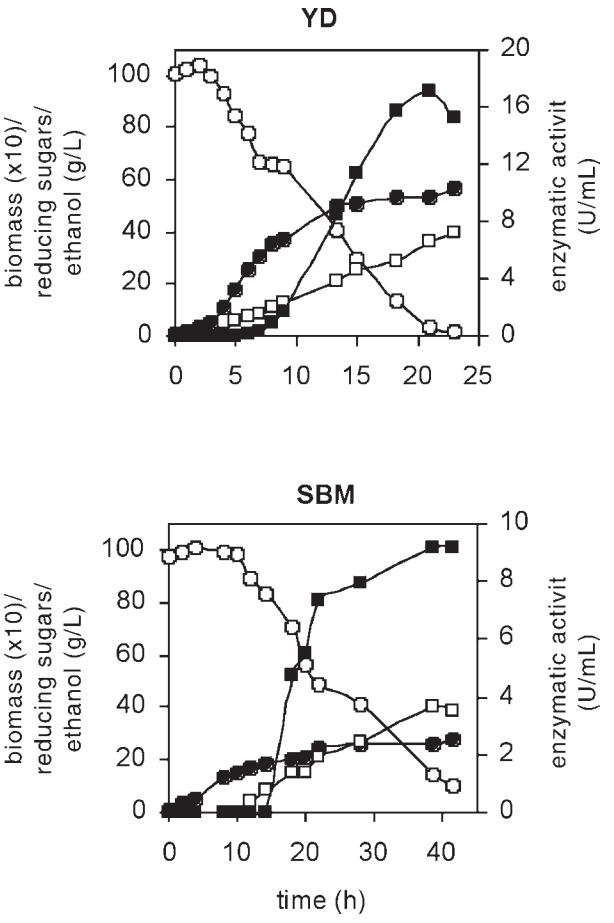

Fig. 1. Kinetics of batch ethanol-PG coproduction in YD and SBM media (○) Reducing sugars; (●) biomass; (□) ethanol; (■) PG.

Table 1
Comparison of Batch Ethanol-PG Coproduction in YD and SBM Media[a]

	YD	SBM
Maximum specific growth rate (μ_m) (h^{-1})	0.562	0.281
Biomass/substrate yield	0.057	0.031
Fermentation efficiency (%)[b]	76.8	95.1
Volumetric ethanol productivity (g/[L·h])[c]	1.94	1.37
Ethanol (g/L)	39.4	40.6
Volumetric PG productivity (U/[mL·h])[d]	0.74	0.24
Maximum volumetric PG productivity (U/[mL·h])	4.8	0.91
Maximum specific PG production rate (U/[mg·h])	1.08	0.46
PG (U/mL)	17.0	9.1
Enzyme/biomass yield (U/mg)	3.2	3.5

[a]All values correspond to the mean of three fermentations.
[b]Related to maximal theoretical ethanol yield on the basis of substrate consumed.
[c]Estimated from ethanol concentration vs time regression line (*see* Fig. 3).
[d]For overall process.

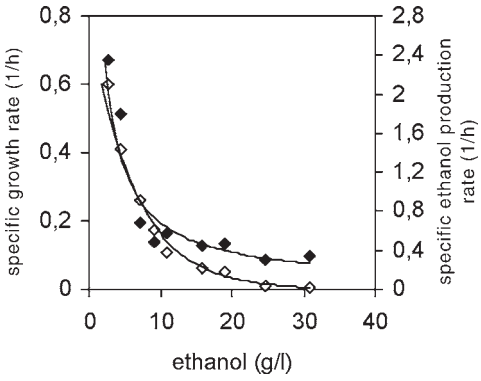

Fig. 2. Influence of endogenously produced ethanol on specific growth (◇) and specific ethanol production (◆) rates for batch fermentation in YD medium.

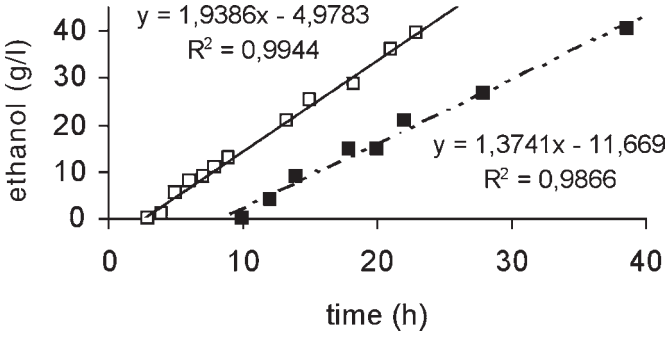

Fig. 3. Ethanol production during course of fermentation in YD (□) and SBM (■) media.

reported value for the *K. marxianus* IMB3 strain *(19)*. According to the smaller biomass conversion, the best fermentation efficiency (95.1%) was achieved with SBM.

Latency intervals of 4 and 12 h in PG production (Fig. 1), corresponding to the latency also seen in ethanol production (Figs. 1 and 3), were observed in the YD and SBM media, respectively, and occurred after a continuous increase in accumulated PG activity until stationary phase had been reached (Fig. 1). The accumulated value of 17 U/mL in YD was almost twice that found with SBM, although the enzyme/biomass yields were similar (Table 1). The volumetric and specific enzyme production rates followed a sigmoid-like trend regarding fermentation time (Fig. 4), with maximum values occurring at 12 (YD) and 18 (SBM) h of fermentation—exactly when specific microbial growth was quite low and accumulated ethanol was close to 18 g/L in both media. PG production was arrested when microbial growth ceased (Fig. 4).

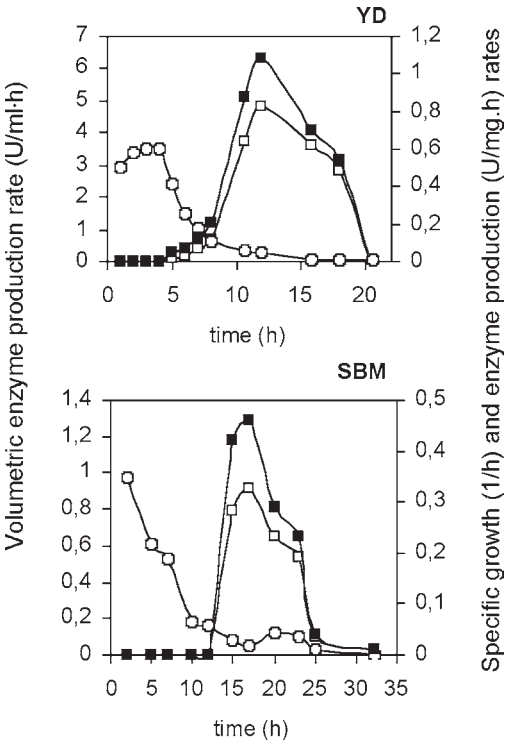

Fig. 4. Dependence of volumetric (□) and specific (■) enzyme production rates on specific growth rate (○) during batch fermentation in YD and SBM media.

Recovery, Characterization, and Exploration of PG's Potential in Food Processing

After centrifugation, crude enzyme from YD medium was concentrated fivefold through an industrial ultrafiltration membrane system that allowed 83% enzyme recovery. Thus, the loss of enzymatic activity was low and mainly took place during the ultrafiltration step, possibly owing to turbulences arising in the process. Product specifications are summarized in Table 2. According to the color intensity and hue, as well as total and volatile fatty acids content of the enzyme concentrates, no deviations in the sensory qualities of the enzymatically treated food or beverage could be expected, although the presence of 5% (v/v) ethanol and yeast flavors must be taken into account. The crude enzyme showed good stability at pHs above 4.5, preserving complete catalytic power for more than 24 h of incubation (Fig. 5). At lower pHs, losses in activity were apparent (Fig. 5).

Table 3 presents the results of the enzymatic treatment of orange juice and pear and apple pulps. A significant twofold increase ($p < 0.01$ for pear and apple; $p < 0.05$ for orange) in filtration speeds was observed for all samples compared with controls, whereas nonsignificant differences ($p > 0.05$) were detected when the incubation time was increased. These results

Table 2
Specifications of PG Enzymatic Concentrate Obtained From Pilot-Scale Batch Production Using YD Medium

Parameter	Value[a]
PG activity (U/mL)	63 ± 4
Specific PG activity (U/mg)	19.6 ± 0.6
PG purity (%)[b]	9
pH_{opt}[c]	4.5
T_{opt} (°C)[c]	55
PG molecular weight (kDa)[c]	41.7
Ethanol (g/L)	39 ± 1
Total acidity (g/L)[d]	4.1 ± 0.1
Volatile acidity (mg/L)	290.1 ± 9.9
Color	Yellow
Luminance (%)	72.7
pH	5.0

[a]Average ± SD of eight determinations.
[b]As fraction of total proteins.
[c]From ref. *13*.
[d]As acetic acid.

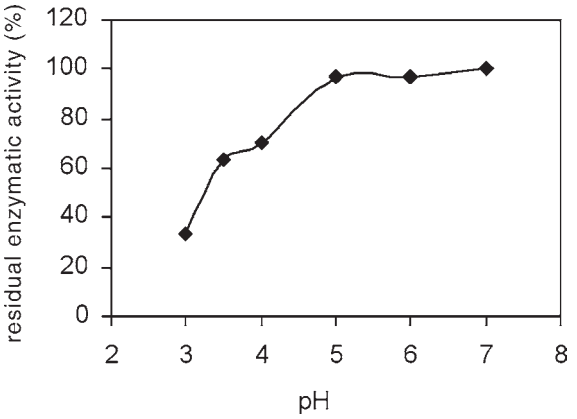

Fig. 5. Influence of pH on enzyme stability of YD fermentation crude.

demonstrate that a 1 h treatment was sufficient and that pectin depolymerization played a key role in this important technological parameter related to the processing of fruit juices.

Another issue addressed here was the potential of yeast endo-PG in the juice clarification. An almost complete clarification (95.3%) was attained for apple juice (Table 3, Fig. 6); in fact, a degree of turbidity similar to that of the commercial clear apple juice (0.060) was obtained, thus confirming the results reported by Gómez-Ruiz et al. *(20)*. Compared with the control, a slight but significant ($p < 0.05$) increase in settled solids (>3%) was

Table 3
Enzymatic Treatment of Orange Juice and Pear and Apple Purees[a]

	Orange		Pear		Apple	
Parameter	C	P	C	P	C	P
Filtration speed (mL/s)						
60 min	2.24 ± 0.30	3.89 ± 0.42	3.45 ± 0.45	8.54 ± 0.96	2.44 ± 0.25	4.42 ± 0.37
120 min	2.24 ± 0.34	3.95 ± 0.35	3.33 ± 0.53	8.33 ± 0.87	2.44 ± 0.21	4.42 ± 0.47
Turbidity (A_{650})						
60 min	0.031 ± 0.004	0.020 ± 0.005	0.364 ± 0.004	0.363 ± 0.004	0.769 ± 0.006	0.035 ± 0.005
120 min	0.031 ± 0.006	0.018 ± 0.004	0.362 ± 0.004	0.364 ± 0.005	0.767 ± 0.007	0.036 ± 0.004
Settled solids (%)						
60 min	—	—	24.41 ± 0.40	19.14 ± 0.30	17.85 ± 0.45	21.02 ± 0.32
120 min	—	—	24.49 ± 0.33	19.11 ± 0.27	17.45 ± 0.32	20.84 ± 0.35

Abbr: C, control, the enzyme was previously heat inactivated; P, problem.
[a]All values correspond to means ± SD of five determinations.

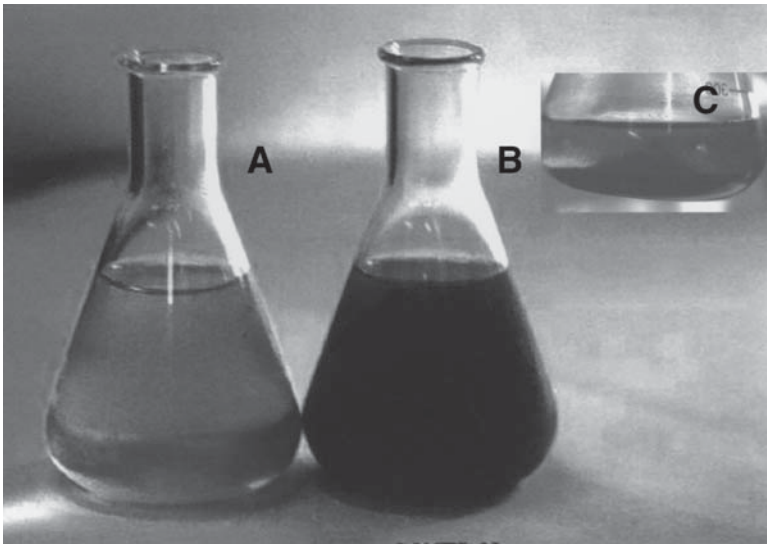

Fig. 6. Appearance of apple juice clarification with *K. marxianus* PG: **(A)** clarified apple juice after treatment with PG; **(B)** same as (A) but the enzyme was previously heat inactivated. The insert **(C)** depicts an intermediate stage in the recovery of oxidized apple juice of (B) by means of *K. marxianus* PG (note flocculation as brown/black sediment).

observed for enzymatically treated apple pulp, in agreement with the degree of clarification achieved. Apple juice cloud is composed of 30–40% proteins that are positively charged at the particular pH of the juice and is associated with polyphenolics. Subsequently, partially hydrolyzed pectin surrounds these structures, leading to the neutralization of electrostatic charges, thereby inducing flocculation *(5)*. As a consequence, the weight of settled solids increases. For orange and pear juices, nonsignificant ($p > 0.05$) changes in turbidity were obtained, in contrast to the increased filtration rate observed. Nevertheless, the mass of settled solids was significantly ($p < 0.01$) diminished (about 22%) in pears, which suggests the possibility of obtaining higher yields of extracted juice by means of yeast PGs.

Regarding the usefulness of yeast PGs for macerating plant tissues, the results can be seen in Figs. 7 and 8. The degree of maceration can be estimated by measuring the loss of weight owing to fluidization when the tissue is exposed to a flow of water. For the experimental conditions assayed, a considerable macerating activity (42.3%) was obtained for cucumber, in agreement with the findings of a previous report *(21)*. Taking into account that Mussell and Morre *(18)* used cucumber as a substrate for quantitative bioassays of PG, this result was to be expected. Regarding carrots and bananas, only 22.3 and 10.0% of macerating activity was obtained, respectively.

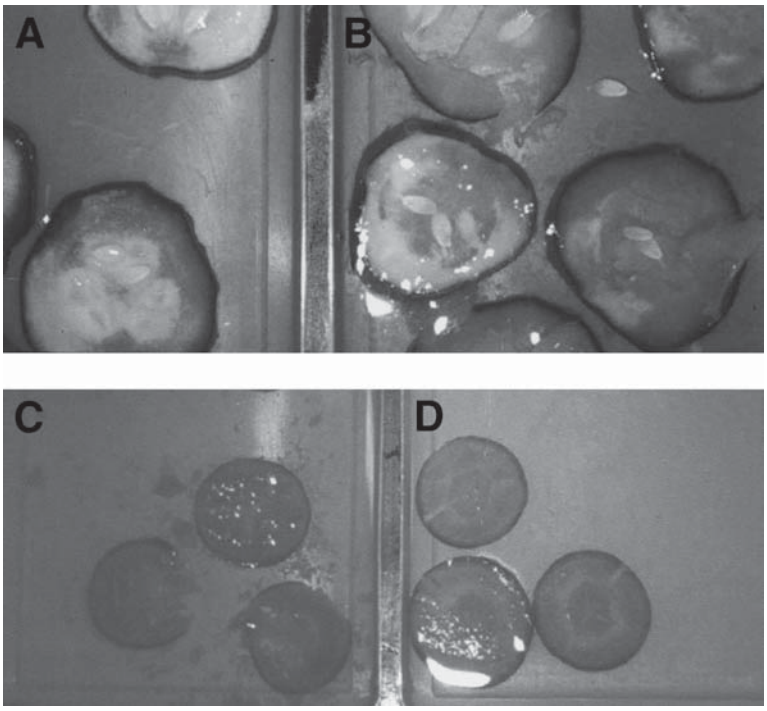

Fig. 7. Tissue softening caused by *K. marxianus* PG on **(B)** cucumber and **(C)** carrot. **(A, D)** Respective controls where enzyme had been previously inactivated.

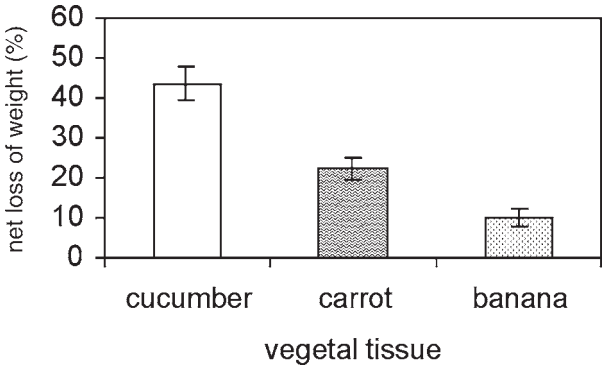

Fig. 8. Net loss of weight in enzymatically treated vegetable tissues when fluidized cells were removed by rinsing with water.

Discussion

Ethanol and PG Production in Batch Processes

A certain inhibition of microbial growth and a lower biomass/substrate yield were observed in SBM medium (Fig. 1, Table 1). These results were expected since furan derivatives such as furfural and hydroxy-

methylfurfural are present in molasses and their growth-inhibiting effect has been previously reported *(22–24)*. The toxic effect of these compounds could, in turn, be involved in higher maintenance costs for the cells, causing the observed lower cell yield in YD. Moreover, because the inoculum came from a yeast extract-based medium, an adaptation in the cell enzymatic machinery is necessary in order for growth to occur. The use in this experiment of gentle stirring at 100 rpm allowed homogeneous biomass distribution in the fermentor, thus avoiding biomass sedimentation as well as the formation of undesirable substrate concentration gradients. This action allowed a considerable reduction in the fermentation time in both of the media compared to previously reported static laboratory cultures *(13)*.

An interesting issue in the present study was that ethanol production took place at an almost constant rate throughout the course of fermentation in both media (Fig. 3), whereas in other instances the usual behavior is a decrease in the ethanol production rate as it accumulates in the extracellular broth owing to its toxic effect. Ethanol productivities in both media and for this particular strain can be considered acceptable for a batch process, where typical values lie between 1.8 and 2.5 g/(L·h). The high ethanol productivity obtained in YD is in agreement with the observed higher specific growth. Nevertheless, a striking fall (Fig. 2) in specific microbial growth and specific ethanol production rates was observed for ethanol concentrations higher than 3 g/L, in agreement with the low ethanol tolerance of *K. marxianus (19,25,26)*. The specific ethanol production rates at ethanol levels higher than 30 g/L observed here (Fig. 2) are considerably higher than those reported by Hack and Marchant *(19)* for the thermotolerant *K. marxianus var. marxianus* IMB3 strain, for which only a negligible ethanol production is observed under such conditions.

Under the conditions studied, PG production in the present strain was characterized by the existence of a latency period at the beginning of the fermentation, during which no PG activity was detected. This initial latency step is probably associated with the existence of certain aerobic conditions in the culture (a small airflow was established at the start and cellular density was still low). Thus, PG synthesis could be partially repressed by oxygen according to previous studies *(13)*. A slight lag in PG secretion for glucose concentrations above 100 g/L has also been observed for other *K. marxianus* strains *(27)*. The accumulated value of 17 U/mL in this pilot experiment is similar to that of 18.8 U/mL reported for laboratory assays with this strain using YNB-glucose medium *(13)*.

Maximum PG synthesis took place when the microorganism was growing slowly and close to the stationary phase. The results are in agreement with those reported by Schwan and Rose *(27)*, who found that *K. marxianus* CCT 3172 secreted approx 90% of the total PG in the early stationary phase. A relationship between growth under oxygen-limited conditions and the induction of PG synthesis seems to exist; this in turn is reinforced by other stress conditions, such as high ethanol concentrations and nutrient starvation. Madhani et al. *(28)* have shown that the *PGU1* gene

is regulated in *S. cerevisiae* by the mitogen-activated protein kinase pathway, which is involved in pseudohyphal differentiation as a response to a reduced availability of essential nutrients. Other yeast glycohydrolases such as invertase show a similar behavior in both wild-type and recombinant *S. cerevisiae* strains *(29,30)*. However, additional studies will be required to confirm this appreciation of PG synthesis under stressing conditions.

The best results regarding the parameters of enzyme production corresponded to the use of YD medium, which supports a more active microbial growth, while the enzyme/biomass yields were similar for both media (Table 1). This latter aspect suggests that, besides anaerobiosis, another determinant factor in PG production could be the number of viable cells in the culture.

The fact that the maximal amount of accumulated enzyme was obtained during the end of active growth would allow the design of a continuous production process specifically directed to ethanol-PG coproduction. Moreover, it is known that ethanol tolerance is higher in continuous processes compared with batch processes. On the other hand, a considerably shorter fermentation time and the possibility of avoiding the difficulties associated with the growth of filamentous microorganisms in industrial fermentors are other advantages supporting the use of yeasts instead of fungi in PG production.

PG's Potential in Food Processing

The results obtained in the evaluation of the enzyme in fruit juice processing suggest that *K. marxianus* PG may have a field of application. In this sense, it is remarkable that the enzymatic action took place in entirely natural juices (without any prior treatment) where pH is low (3.3 for apple juice and 4.5 for pear). In the case of orange juice, an interesting result was that enzymatic treatment led to an improved filtration rate without affecting the turbidity of natural orange juice, an important quality parameter that must be preserved during juice processing.

Regarding macerating activity in vegetable tissues, the relative small differences observed between the control and the problem in bananas may be owing to the existence of a significantly high endogenous PG activity, since a high level of maceration was observed in the controls (data not shown). In banana, it appears that pectinase plays a dominant role in softening during ripening according to the results reported by Prabha and Bhagyalakshmi *(31)*. In the case of carrots, the use of pectolytic preparations with a high level of protopectinase activity *(32)* is recommended in order to solubilize the protopectin present in these tissues. The absence of protopectinase activity in the crude enzyme studied would explain the low degree of maceration in carrots. On the basis of these results, application of this enzyme in macerating remains limited.

In conclusion, the results of our study suggest that the *K. marxianus* strain CCEBI 2011 would be a good candidate for the industrial coproduc-

tion of PG and ethanol. Although production levels of pectic enzymes in industrial processes led by *Aspergillus* are higher than the reported here, it must be taken into account that production in yeast is susceptible to being carried out in a continuous manner, where PG would be the byproduct of ethanol production, thus contributing to improvement of the economic efficiency of the whole bioprocess.

Studies concerning the improvement of ethanol tolerance of the strain and optimization of the culture conditions and medium composition are currently under way. Nevertheless, the potential of the enzyme in food processing is clear, although it could be improved.

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