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Growth and protopectinase production of *Geotrichum klebahnii* in batch and continuous cultures with synthetic media†

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***Geotrichum klebahnii* ATCC 42397 produces a protopectinase (PPase-SE) with polygalacturonase (PGase) activity. The microorganism was aerobically cultivated in synthetic media. Glucose, fructose and xylose yielded the highest enzyme levels (10–11 PGase units ml^{-1}). Galacturonic acid repressed enzyme production and no growth was obtained with disaccharides and pectin. Specific enzyme activity obtained in an O_2-limited culture was similar to that found in nonlimited ones. A growth yield ($Y_{x/s}$) of 0.49 g of cell dry weight per gram of glucose consumed was obtained in a typical batch bioreactor culture. Enzyme production was growth associated, and no major products other than biomass and CO_2 were detected. The volumetric enzyme activity reached a maximum around D=0.3–0.4 h^{-1} in glucose-limited continuous cultures. However, it varied strongly (together with microorganism morphology) even after retention times ≥8 at any D tested (0.035–0.44 h^{-1}) though the rest of the culture variables remained fairly constant. No correlation between morphology and enzyme activity could be obtained. Enzyme production was poor in urea- and vitamin-limited continuous cultures. In all cases, biomass and CO_2 accounted for ≅100% of carbon recovery though $Y_{x/s}$ values were different.** *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 260–265.

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Introduction

Protopectin is the water-insoluble parental pectic substance that occurs in plant tissues and yields soluble pectin upon restricted hydrolysis [27]. The enzymatic solubilization of pectin from protopectin is carried out by a heterogeneous group of enzymes named pectin-releasing or pectin-solubilizing enzymes (hereafter called protopectinases, PPases). The first Ppase reported was found in a culture filtrate of a yeast-like fungus (*Trichosporon penicillatum*, strain SNO-3) and named a PPase-SE [22]. This strain was reclassified as *Geotrichum penicillatum* and later as *G. klebahnii* (ATCC 42397).

Ppase-SE has been isolated, partially characterized and identified as an endopolygalacturonase (endo-PGase, EC 3.2.1.15) [24,25]. This enzyme is highly efficient for enzymatic extraction of pectin from citrus peel [23] and for the maceration of potato tissues to obtain single-cell foods [17].

In the reported studies on Ppase-SE, the producing strain was cultivated in a complex culture medium composed of yeast extract, glucose and peptone [22–25]. Therefore, information on the nutritional requirements of the strain and on the effect of medium components on enzyme expression is scarce. Here, we describe microbial growth and Ppase-SE expression in different synthetic media in both batch and continuous cultures.

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Materials and methods

Chemicals

D-Galacturonic acid monohydrate (GALA), polygalacturonic acid (PGA) from orange and citrus pectin were from Sigma (St. Louis, MO, USA). All other chemicals used were commercially available and of analytical grade.

Microorganism, media, inocula and culture conditions

G. klebahnii ATCC 42397 was used. Permanent cell stocks, in 15% (v/v) glycerol, were stored at −70°C. When necessary, cells were streaked and grown in a maintenance medium (MM) with the following composition (per liter): 5 g meat peptone (Difco), 5 g yeast extract (Difco), 20 g glucose, and 20 g agar, Ph 5.0, at 30°C, and kept at 4°C under a layer of vaseline for 6 months at most.

A reference medium (RM), developed in our laboratory, was also used and it contained (per liter): 5 g glucose (limiting substrate), 1 g K_2HPO_4, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.6 g urea, 0.45 g citric acid, 1 ml vitamin solution, 1 ml micronutrient solution C, and 1 ml micronutrient solution A, pH 5.0.

The vitamin solution contained (per liter): 6 mg folic acid, 6 mg *myo*-inositol, 6 mg D-biotin, 0.8 g calcium pantothenate, 0.8 g *p*-aminobenzoic acid, 0.8 g riboflavin, and 1.6 g pyridoxine. Mineral micronutrient solution C contained (per liter): 0.6 g citric acid, 0.15 g $CoCl_2$, 3 g $MnSO_4 \cdot H_2O$, 5 g $ZnSO_4 \cdot 7H_2O$, 15 g $FeSO_4 \cdot 7H_2O$, and 0.75 g $CuSO_4 \cdot 5H_2O$, pH 1.5. Mineral micronutrient solution A contained (per liter): 0.65 g $Na_2MoO_4 \cdot 2H_2O$, 0.1 g KI, and 0.1 g H_3BO_3, pH 1.5.

In some experiments, glucose in the RM was replaced by different carbon and energy sources (at carbon concentrations equivalent to 5 g l^{-1} of glucose): D-xylose, D-galactose, D-

fructose, D-lactose, maltose, sucrose, glycerol, D-mannitol, D-sorbitol, acetic acid, DL-lactic acid, GALA and pectin.

Modified RM media were used in continuous cultures carried out with substrate limitations other than glucose. The corresponding limiting substrate concentrations in these media were (in each case other component concentrations were those mentioned for RM): (a) phosphate-limited medium: 0.043 g l^{-1} K_2HPO_4; (b) urea-limited medium: 0.075 g l^{-1} urea; (c) vitamin-limited medium: 0.010 ml l^{-1} vitamins solution.

All components of media were autoclaved (121°C, 15 or 20 min for Erlenmeyer or bioreactor cultures, respectively) except in the cases of urea and vitamins, which were sterilized separately by filtration through a cellulosic filter paper (0.22 μm, E02WP02500, MSI, Westboro, MA, USA).

Cultures were inoculated with an appropriate dilution of a suspension (in 20 mg l^{-1} Tween 80) of the microorganism grown in MM at 30°C for 48 h. Cells were washed to eliminate all remaining substances derived from MM. Initial OD_{620} was around 0.2 in both flasks and bioreactor cultures.

Batch cultures were run in triplicate 500-ml Erlenmeyer flasks containing 50 ml of medium (oxygen-sufficient cultures) or 400 ml of medium (oxygen-limited cultures), at 30°C, on a rotary shaker at 200 rpm. The microorganism was also cultivated batchwise in a 4-l LKB bioreactor (Ultroferm 1601, Bromma, Sweden) with 3 l of medium, at 30°C, with aeration (0.2 vvm) and agitation (500 rpm). A pH=5.0±0.2 controlled batch culture was also carried out under the same conditions but using an RM with GALA (5 g l^{-1}) as carbon and energy source.

Continuous cultures were carried out in an LH bioreactor (SET 002, Inceltech, France) at 30°C, with aeration (0.2 vvm) and agitation (650 rpm), in 1000 ml of medium. Culture pH was automatically controlled at 5.0±0.2. Feeding of fresh medium was done by a peristaltic pump (LKB, Bromma, Sweden). Dilution rates (D) assayed ranged from 0.035 to 0.44 h^{-1}.

Culture pH was controlled automatically with 1 N H_2SO_4 or 1 N NaOH. Foam was automatically controlled in batch and continuous cultures, when needed (occasionally), by addition of a 10% (v/v) antifoam (1520, Dow Corning, MI, USA) solution. Dissolved oxygen was measured with an amperometric or polarographic type electrode for the LKB and LH bioreactors, respectively. Outlet gas was analyzed with a paramagnetic O_2 detector (Series 1100, Servomex, USA) and an infrared CO_2 detector (Pir 2000, Horiba, Japan). The O_2 uptake and CO_2 production rates were calculated according to Cooney *et al.* [8].

Analytical techniques

Biomass content in Erlenmeyer-flask cultures was determined by measurement of the optical density at 625 nm (OD_{625}), and the values reported are means±SD of values from three flasks. In the case of samples from bioreactor cultures, biomass was evaluated by dry weight determinations. For batch cultures, 25-ml samples were used. Samples from continuous cultures (15 ml up to D=0.12 h^{-1} and 25 ml for higher values of D) were collected from the effluent on an ice bath. These samples were filtered through a cellulosic filter paper (0.45 μm, pore size E04WP04700, MSI, USA); the biomass retained was washed carefully with distilled water, and dried at 80°C to constant weight.

Samples for glucose and enzyme analysis were immediately centrifuged in an Eppendorf microfuge (18,000×g, 2 min, 5°C). The supernatant was carefully separated with a pipette and frozen (at −20°C) until used for quantification. In the case of continuous cultures, samples (3–4 ml) were taken from the effluent, collected on an ice bath and processed as above.

Sampling in continuous cultures carried out at different D values, was done as follows: for D values up to 0.12 h^{-1} samples (3) were taken between six and eight retention times; for D values higher than 0.12 h^{-1} samples (3) were taken between 10 and 17 retention times, approximately.

Glucose was determined with the glucose oxidase–peroxidase (Glicemia, Wiener, Argentina) method. Other carbohydrates were analyzed by the anthrone method [15]. Ammonium was quantified by the indophenol blue reaction [9].

Enzyme analysis

Enzyme activity of Ppase-SE was quantified by using either an insoluble substrate (protopectin) or a soluble substrate (PGA) depending on the carbohydrate content of the samples, as follows:

Technique (a): The protopectinase activity of Ppase-SE was determined with a modification of the technique previously reported [6], in sets of samples containing predominantly high sugar concentrations. In the modification the pectic substances solubilized from protopectin were quantified by heating the sample at 100°C in a concentrated sulfuric acid/tetraborate solution followed by addition of 3-phenylphenol according to the method developed by Blumenkrantz and Asboe-Hansen for the specific determination of uronic acids [4]. One unit of PPase activity was defined as the activity that releases soluble pectic substances corresponding to the reducing power of 1 μmol of GALA at 37°C per minute.

Technique (b): The PGase activity of PPase-SE was carried out, particularly in sets of samples with low sugar concentrations, and expressed as PGase units. PGase activity was assayed according to Cavalitto *et al.* [7]. One unit of PGase activity was defined as the activity that releases a reducing power equivalent to 1 μmol of GALA at 37°C per minute.

Technique (a) is not convenient for routine analysis whereas in technique (b) residual amounts of carbon and energy sources from the culture medium interfere in the determination causing high blank values. Therefore, enzyme activity was determined with technique (a) in those samples with a high carbohydrate content and with technique (b) in all other cases.

The quotient between PPase and PGase activity ($q_{PPase/PGase}$) for PPase-SE was reported to be 0.076 [7]. Therefore, for practical purposes, figures obtained with technique (a) were converted into and expressed as PGase units (values are means±SD of three replicates).

Results and discussion

Flask cultures

Cultures carried out in Erlenmeyer flasks with 50 ml of RM showed that the microorganism grew well in this medium, reaching a final OD_{625} of 8.3, which corresponds to ≅2.5 g l^{-1} of dry weight, and yielding an extracellular enzyme activity of 11.8±0.8 PGase units ml^{-1}. pH values remained almost constant during most of the culture. Glucose was exhausted when the culture attained the stationary growth phase (22 h), at which time the pH started to increase suddenly. When glucose concentration in the RM was increased by 20%, the final dry

weight changed proportionally. Therefore, we confirmed that RM was limited in the carbon source.

Preliminary experiments showed that enzyme activity declined when culture pH started to increase above pH 7. It was reported that PPase-SE is stable in acidic pHs, whereas the enzyme stability strongly declines above this value [25]. Therefore, processes were stopped when the pH approached neutrality to avoid enzyme inactivation.

The effect of different carbon and energy sources other than glucose on biomass growth and enzyme production was tested using modified versions of RM. The final biomass levels attained are shown in Figure 1. The microorganism assimilated and grew on all the monosaccharides tested (D-xylose, D-galactose, and D-fructose) and on glycerol. In the cases of GALA and DL-lactic acid, final levels of biomass were around 50% and 20%, respectively, of that achieved in the RM. Growth was negligible with all disaccharides used (D-lactose, maltose, and sucrose) and with D-mannitol, D-sorbitol, acetate and pectin.

Figure 1 also shows the PGase activity values attained at the end of culture with all carbon sources that supported growth. From these experiments, it can be concluded that the best sources for PPase-SE production are glucose, fructose and xylose, which yielded similar final enzyme activities. In general, extracellular depolymerizing enzymes such as pectinases, and, in particular, polygalacturonases share a common feature, which is their glucose-repressible synthesis. This effect has been reported in *Saccharomyces cerevisiae* [2,3], *Cryptococcus albidus* [10] and *Kluyveromyces marxianus* [26]. Moreover, it was also reported that fructose delayed enzyme secretion in *S. cerevisiae* [2]. By contrast, *G. klebahnii* does not exhibit both effects at all, and PPase-SE seems to show constitutive synthesis and secretion mechanisms.

Results obtained using GALA as carbon and energy source suggest a strong repression effect on enzyme expression. However, because of GALA consumption, pH increased rapidly to values where the enzyme is unstable. The GALA repression effect was confirmed in pH-controlled batch cultures (see below). The

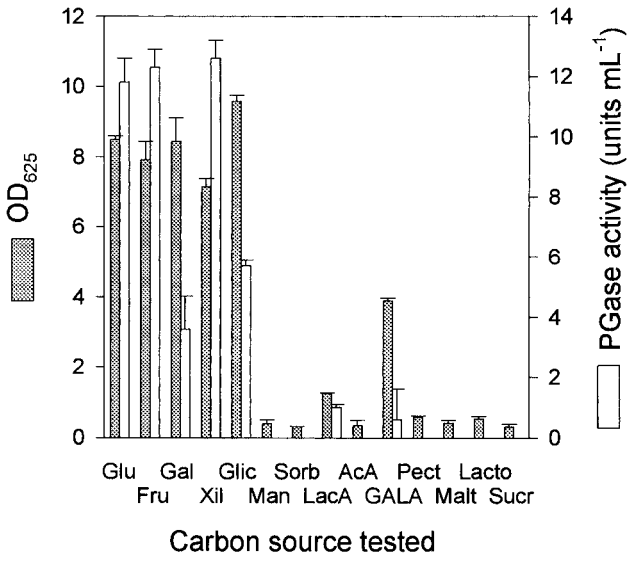

Figure 1 Effect of different carbon and energy sources on final biomass level (as OD_{625}) and PPase-SE activity (as PGase units per milliliter) in shake flask cultures of *G. klebahnii*.

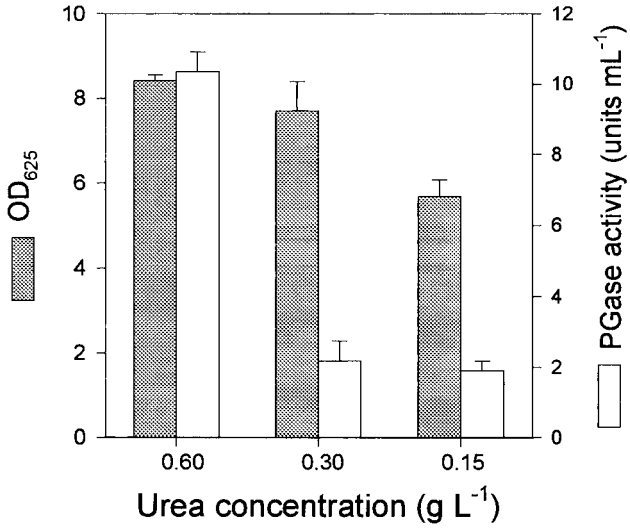

Figure 2 Effect of urea concentration on final biomass level (as OD_{625}) and PPase-SE activity (as PGase units per milliliter) in shake flask cultures of *G. klebahnii*.

repression effect of GALA on enzyme expression is opposite to the behavior of several yeast strains such as *S. cerevisiae* [2] and *C. albidus* [10] in which GALA has been described as an inducer.

The effect of urea concentration in RM was studied and results are shown in Figure 2. When urea concentration was reduced from 0.60 (RM) to 0.30 and to 0.15 g l^{-1}, culture pH remained almost constant at about pH 5 during the whole process and no free ammonium was detected. The final level of biomass achieved was not proportionally reduced, but enzyme activity was seriously affected reaching in both cases around 20% of the value achieved with the original RM. This clearly demonstrates that an excess of nitrogen source in the culture medium is needed for good enzyme production.

The carbon source concentration in RM (5 g l^{-1}) and the ratio of medium volume to flask volume (1/10) in the above-described experiments were chosen to conduct the cultures without oxygen limitations. To test oxygen-limited conditions on cell growth and enzyme production, cultures were also run with 400 ml of medium in 500-ml Erlenmeyer flasks. Under these conditions, biomass and enzyme production were lower (OD_{625}=4.5 and 6.5±0.6 PGase units ml^{-1}, respectively, at 22 h of culture) than those observed in nonlimited-oxygen cultures. Nevertheless, the specific activity (PGase units OD^{-1}) was similar to that found in nonlimited-oxygen cultures. This result is similar to those found in *C. albidus* [10] and *Geotrichum lactis* [19] in which high oxygen supply does not seem to be crucial for enzyme synthesis. In contrast, in *K. marxianus* [1,26], *Kluyveromyces fragilis* [13], *Kluyvermyces lactis* [16], *Saccharomyces fragilis* [28] and *Saccharomycopsis fibuligera* [11], low or null oxygen concentrations seem to be a requisite for enzyme synthesis. On the other hand, a certain type of volatile product(s) with an aroma resembling apple was produced during the second half of the oxygen-limited culture. Pastore *et al.* [20] mentioned the production of a fruity aroma in the culture of a *Geotrichum* sp. grown in shake flasks with a medium containing 50 g l^{-1} of carbon source. We assume that these culture conditions result in oxygen-limited growth. According to these results, aroma production in these two strains seems to be associated with oxygen-limited cultures, a growth condition in which the

microorganism would shunt part of the carbon source to the synthesis of volatile compounds.

Bioreactor cultures

Figure 3 shows the time course of a typical batch culture of *G. klebahnii* in RM. From these data, an approximate maximal specific growth rate (μ_m) of 0.47 h^{-1} was calculated, a value typical for yeasts. Culture pH remained constant at around pH 5.5 until the carbon source was exhausted, at which time it started to increase until the end of culture (data not shown). The total glucose consumed was 4.91 g l^{-1} and the biomass produced was 2.39 g l^{-1}, values that lead to a biomass yield ($Y_{X/S}$) of 0.49 g^{-1}, a typical figure for a fully aerobic metabolism of carbohydrates. Enzyme synthesis seems to be growth associated, reaching 14.3 PGase units ml^{-1} at the end of culture. On the other hand, peaks of O_2 consumption rate (r_{O_2}) and CO_2 production rate (r_{CO_2}), both around 25 mmol l^{-1} h^{-1}, took place at around 23 h of culture and then suddenly declined simultaneously with glucose exhaustion. r_{CO_2} paralleled r_{O_2}, indicating that the respiratory quotient remained constant, being close to 1. Dissolved oxygen concentration was always above 30% saturation (data not shown), ensuring non oxygen limitation of the culture. Total O_2 consumption and CO_2 production were 79.2 and 76.7 mmol l^{-1}, respectively. With these figures, the C-recovery of the culture was calculated as 99.8%. Indicating that no other products besides biomass and CO_2 were formed (in this case, the carbon contribution of the enzyme to the carbon balance is negligible). These results indicate that *G. klebahnii* can be regarded as a highly efficient biomass producer.

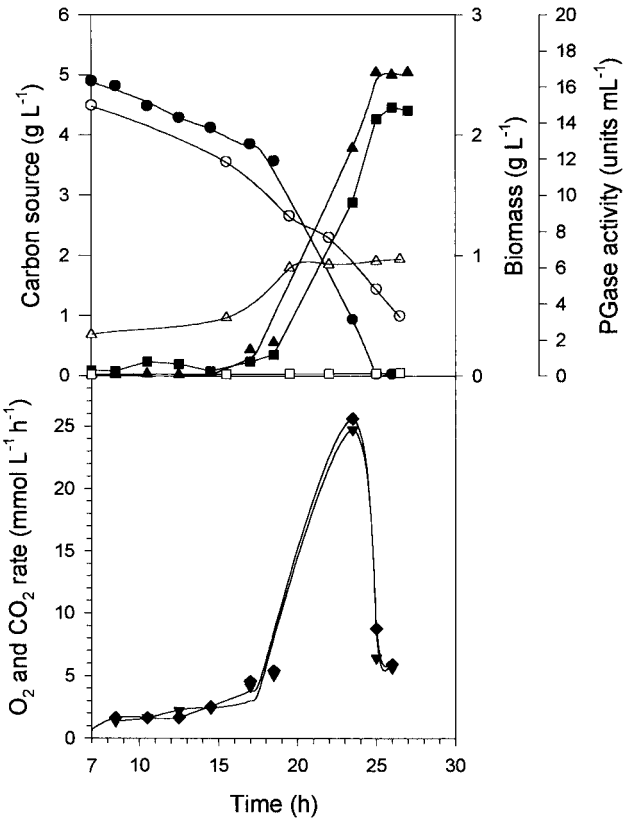

Figure 3 Time course of biomass, enzyme activity, residual carbon source and O_2 consumption and CO_2 production rates in a 4-l bioreactor batch culture of *G. klebahnii* in RM either with glucose (filled symbols) or GALA (empty symbols) as carbon source. (●, ○) Residual carbon source; (▲, △) biomass; (■, □) PGase activity; (◆) O_2 consumption rate; (▼) CO_2 production rate. Lag growth phase is partially shown.

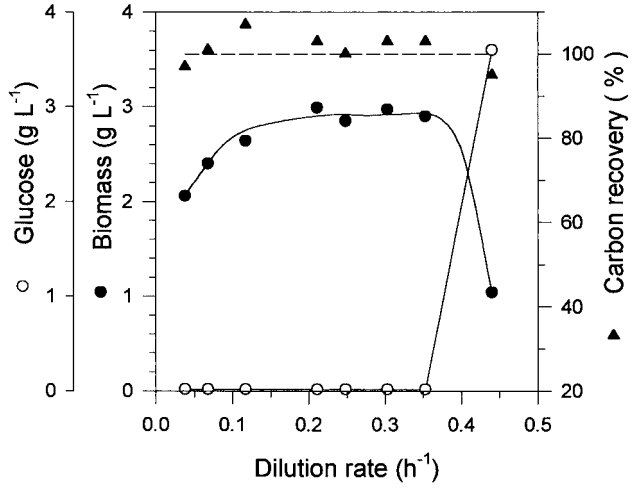

Figure 4 Steady-state values for residual glucose, biomass and carbon recovery in continuous culture of *G. klebahnii* growing in RM at different dilution rates.

A pH-controlled batch culture was carried out to verify the effect of GALA as carbon and energy source (Figure 3). GALA was not completely consumed (final concentration = 1.00 g l^{-1}). As no enzyme activity was detected during the whole culture, we concluded that GALA is a potent repressor of PPase-SE expression.

Raw data obtained at different D values in continuous cultures carried out with the RM are shown in Figure 4. The variation in dissolved oxygen, biomass content, r_{O_2} and r_{CO_2} observed after four to five retention times for all D values tested were, in all cases, lower than 4% indicating that the cultures achieved the steady state. Glucose concentrations remained at very low values (< 10 mg l^{-1}) up to $D = 0.35$ h^{-1}, increasing thereafter, in accordance with the observed decrease in biomass. Carbon recovery (considering CO_2 plus biomass) remained close to 100% for all D values tested, as previously observed for batch cultures. Therefore, we concluded that a fully oxidative metabolism takes place irrespective of the growth rate.

In general, volumetric enzyme activity increased with D, reaching the highest value (≅40 PGase units ml^{-1}) around $D = 0.30–0.35$ h^{-1}, and then it decreased simultaneously with biomass concentration when the culture was approaching the washing out (Figure 5). However, contrary to the process variables mentioned above, volumetric enzyme activity varied strongly between samples taken at the same D, but at different retention times as can be seen from the standard deviation values shown in Figure 5. A similar result was observed in another continuous culture carried out under the same conditions from $D = 0.20$ up to 0.45 h^{-1}. Microscopic observations of culture samples taken at the same D, but at different retention times showed great variation in the microorganism's morphology from predominantly filamentous forms to predominantly single-cell forms, though biomass concentrations remained fairly constant. In other cases, the morphology change was in the opposite direction. This unexpected

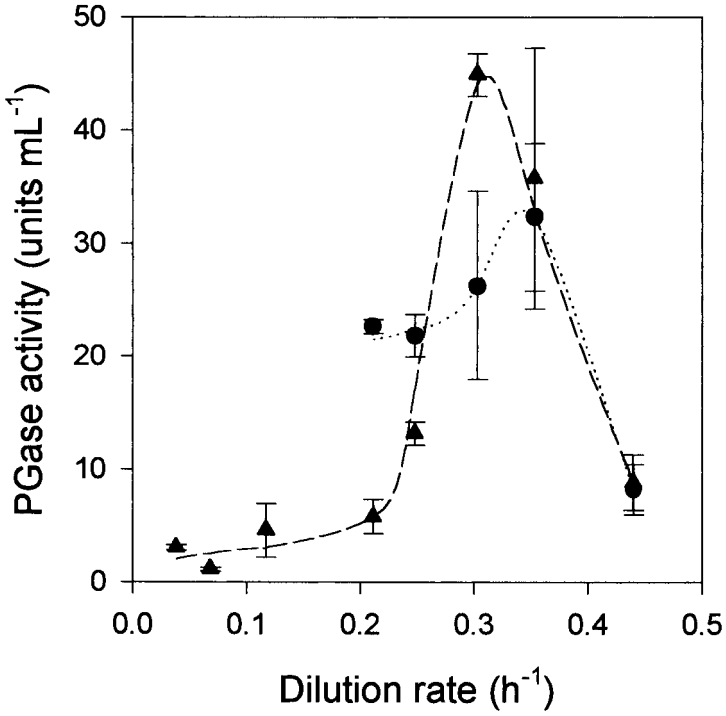

Figure 5 Effect of dilution rate on PPase-SE activity (as PGase units per milliliter) in two independent continuous cultures of *G. klebahnii* growing in RM. (▲) and (●) Continuous culture A and B, respectively.

behavior of the strain could explain, in a first approach, the great variability found in enzyme activity, if one assumed that enzyme production is related to the microorganism's morphology. This phenomenon has already been reported in pullulan production by *Aureobasidium pullulans* [14]. Nevertheless, as result of subsequent experiments, we could not associate the variations in enzyme activity to the changes in microbial morphology (see below).

Table 1 shows the results obtained under different nutritional limitations at $D=0.2\ h^{-1}$. These results indicate that *G. klebahnii* does not exhibit the so-called overflow metabolism [18] because biomass and CO_2 produced account for around 100% of carbon recovery, no matter the type of nutritional limitation. In this sense, this microorganism behaves as a glucose-insensitive one [12], which means that glucose uptake is controlled by the growth rate irrespective of the limiting substrate.

Table 1 also shows that $Y_{X/S}$ under urea and glucose limitations were similar, whereas $Y_{X/S}$ was significantly lower in phosphate and vitamin limitations. In the case of vitamin limitation, one (or more) vitamin(s) is/are the real growth-limiting factor(s). If no products were formed apart from biomass and CO_2, a reasonable explanation for these $Y_{X/S}$ decrements would be a reduction in efficiency of carbon source utilization. Such a type of reduction can be due to: either (i) a reduced efficiency in biomass synthesis (less biomass can be synthesized from a given amount of ATP, therefore lower $Y_{X/ATP}$) or (ii) a reduced energetic efficiency in the respiratory chain (lower P/O ratio). In our case, biomass was always synthesized from the same precursors because the components in all culture media used in these experiments were the same, independent of the nature of the limiting substrate. Therefore, it would be reasonable to assume that $Y_{X/ATP}$ was the same for the different nutritional limitations. Consequently, the reduction found in $Y_{X/S}$ would be the consequence of a lower energetic efficiency.

In a fully oxidative metabolism, the amount of ATP produced in the respiratory chain is proportional to oxygen consumption, so oxygen consumption can be viewed as a measure of the energy requirement for biomass synthesis [5]. Table 1 shows that the amount of biomass produced per gram of oxygen consumed ($Y_{X/O}$) is lower in the cases of phosphate- and vitamin-limiting conditions, which suggests that in these cases the P/O ratios were lower than in glucose- or urea-limited cultures. It has been reported that the loss of one site of oxidative phosphorylation in

Table 1 Effect of nutritional limitations on growth parameters and specific PPase-SE activity in continuous culture of *G. klebahnii* ($D=0.2\ h^{-1}$)

Limiting substrate	$Y_{x/s}$ ($g\ g^{-1}$)	$Y_{x/o}$ ($g\ g^{-1}$)	Specific enzyme activity ($U\ g^{-1}$)	Steady-state remaining glucose ($g\ l^{-1}$)	Carbon recovery (%)
Glucose	0.56±0.013	1.30±0.088	8.50±2.90	0.015	99
Urea	0.54±0.009	1.34±0.039	0.82±0.65	2.83	104
Phosphate	0.51±0.007	1.04±0.063	11.3±3.53	2.81	98
Vitamins	0.48±0.021	0.93±0.086	0.12±0.06	2.80	101

the respiratory chain for yeast growing in sulfate-limited continuous culture [21], which could explain our results.

Specific enzyme activity, as enzyme units per gram of biomass dry weight (units g^{-1}) was also affected by the kind of nutritional limitation (Table 1). In urea- and vitamin-limited cultures, enzyme production decreased dramatically compared to glucose- and phosphate-limited cultures. Again, enzyme activity varied strongly between samples taken at different retention times. In addition, morphological variations, were observed in all nutritional limitations. This suggests again a microbial morphology/enzyme production relationship. However, we could not confirm this type of relationship. Research is in progress to clarify this point.

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