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Localization of Inulinase and Invertase in *Kluyveromyces* Species

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In vivo hydrolysis of inulin and sucrose was examined in selected yeasts of the genus *Kluyveromyces*. Cells, grown in sucrose-limited chemostat cultures, were subjected to treatments for the removal of inulinase, the enzyme responsible for the hydrolysis of both inulin and sucrose. The effects of these treatments were studied by measurement of inulin-dependent and sucrose-dependent oxygen consumption by cell suspensions. In *Kluyveromyces marxianus* var. *marxianus*, inulinase was partially secreted into the culture fluid. Removal of culture fluid inulinase by washing had no effect on sucrose-dependent oxygen consumption by this yeast. However, this treatment drastically reduced inulin-dependent oxygen consumption. Treatment of washed cells with sulfhydryls removed part of the cell wall-retained inulinase and reduced inulin-dependent oxygen consumption by another 80%. Sucrose-dependent oxygen consumption was less affected, decreasing by 40%. Cell suspensions of *K. marxianus* var. *drosophilarum*, *K. marxianus* var. *vanudenii*, and *Saccharomyces kluyveri* rapidly utilized sucrose but not inulin. This is in accordance with the classification of these yeasts as inulin negative. Supernatants of cultures grown at pH 5.5 did not catalyze the hydrolysis of inulin and sucrose. This suggested that these yeasts contained a strictly cell-bound invertase, an enzyme not capable of inulin hydrolysis. However, upon washing, cells became able to utilize inulin. The inulin-dependent oxygen consumption further increased after treatment of the cells with sulfhydryls. These treatments did not affect the sucrose-dependent oxygen consumption of the cells. Apparently, these treatments removed a permeability barrier for inulin that does not exist for sucrose. Nondenaturing polyacrylamide gel electrophoresis and determination of the S/I ratio (relative activity with sucrose and inulin) of enzyme preparations proved that in these yeasts, as in *K. marxianus* var. *marxianus*, hydrolysis of sucrose and inulin is catalyzed by the same enzyme, namely inulinase. This cryptic inulinase activity is not a physiological artifact. When cells were inoculated in media of pH 4.5 and incubated at 35°C instead of the standard cultivation conditions used in yeast taxonomy (pH 5.6, 25°C), rapid growth on inulin occurred. Both inulin- and sucrose-hydrolyzing activities could be detected in culture supernatants of these yeasts under these new conditions. Physiological, ecological, and taxonomic aspects of the occurrence and localization of inulinase in *Kluyveromyces* strains are discussed.

Snyder and Phaff (22) first described the production of inulinase by *Saccharomyces fragilis*, a yeast now known as *Kluyveromyces marxianus* var. *marxianus*. The hydrolysis of inulin, a fructose polymer, can also be catalyzed, albeit very slowly, by invertase (EC 3.2.1.26). The separate classification of these two enzymes has been disputed (2, 15). The main question is whether inulinase should be regarded as a special type of invertase or as a different enzyme with an analogous mode of action.

We recently studied the biochemistry of the inulinase of *K. marxianus*. From this study it appeared that, in addition to substrate specificity, considerable structural differences also exist with the invertase of *Saccharomyces* var. *marxianus cerevisiae* (19). Inulinase secreted into the culture fluid has a molecular mass of about 165 kDa, consists of two protein subunits, and contains 34% of its mass as carbohydrate. The inulinase associated with the cell wall has the same carbohydrate content but is a tetramer with an average size of 350 kDa. In *Saccharomyces cerevisiae*, invertase is secreted in the culture fluid as a dimer with a molecular mass of 270 kDa, whereas the enzyme retained in the cell wall is an octamer of about 800 kDa. Both invertase forms contain up to 50% carbohydrate (10). Little homology was found in the amino acid sequences of the amino-terminal ends of invertase and inulinase (19). In view of the above-mentioned structural differences and the low activity of invertase with inulin as a substrate (11, 23, 27), the separate classification of inulinase and invertase in yeast strains seems justified.

Secreted invertase of *S. cerevisiae* resides mainly in the cell wall, where it performs its physiological function, i.e., the cleavage of sucrose which diffuses into the cell wall (1, 5). From an ecological point of view, the retention of invertase in the cell wall may be beneficial. In this way an efficient scavenging of the hydrolysis products can be accomplished. Similarly, the retention of inulinase in the cell wall of *K. marxianus* may be advantageous for sucrose utilization by this yeast. However, this does not hold for inulin utilization, since inulin cannot penetrate the cell wall (18, 21) and must therefore be hydrolyzed outside the cell wall. The aim of the present study was to compare *Kluyveromyces* and *Saccharomyces* strains with respect to the localization of sucrose-hydrolyzing activities and to study to what extent inulin consumption depends on the presence of inulinase in the culture fluid.

MATERIALS AND METHODS

Yeast strains and growth conditions. *K. marxianus* var. *drosophilarum* CBS 2103, *K. marxianus* var. *marxianus* CBS 6397 and CBS 6556, *K. marxianus* var. *lactis* CBS 683, CBS 739, CBS 1067, CBS 2359, and CBS 8043, *K. marxianus* var. *vanudenii* CBS 5669, *Kluyveromyces lodderi* CBS 2758; *Kluyveromyces waltii* CBS 6430, and *S. cerevisiae* CBS 8066 were obtained from the Yeast Division of the Centraalbureau

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voor Schimmelcultures, Delft, The Netherlands. *Saccharomyces kluyveri* UCD 51-242 was a gift from H. J. Phaff, University of California, Davis, Calif. Yeasts were maintained on YEPD (10 g of yeast extract, 10 g of Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 20 g of glucose, each per liter of demineralized water) agar slopes. Batch cultivation was done at various pH values and temperatures in 50-ml shake flasks containing either Yeast Nitrogen Base (Difco) (5 g liter^{-1}) or mineral salts medium (7) as the source of vitamins and minerals, supplemented with 10 g of carbon substrate liter^{-1} (sucrose, raffinose, maltose, or inulin).

Chemostat cultivation was performed in laboratory fermentors (Applikon, Schiedam, The Netherlands) with a working volume of 1 liter at a dilution rate of 0.1 h^{-1} at 33°C and an oxygen concentration that was 50 to 70% of air saturation. Dissolved oxygen was measured with a polarographic oxygen electrode (Ingold, Urdorf, Switzerland), and pH was controlled by the automatic addition of 1 M KOH at pH 5.5 unless mentioned otherwise. A mineral medium described by Bruinenberg et al. (7) was used, except that the $NaMoO_4 \cdot 2H_2O$ concentration was increased 10-fold. For carbon- and energy-limited growth, sucrose was added to the mineral salts medium to give a final concentration of 10 g liter^{-1}.

Measurement of substrate-dependent oxygen consumption. Inulin, sucrose, fructose, and glucose metabolism by suspensions of intact cells was assayed by following the rate of oxygen consumption with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) in a reaction volume of 4 ml, with a final cell concentration of 0.5 mg of cell (dry weight) ml^{-1} at 33°C. Cell suspensions from sucrose-limited continuous cultures were assayed in three ways: (i) directly after diluting with mineral medium (pH 5.5); (ii) after removal of culture fluid by centrifugation (4,000 × *g*), washing of the cells with mineral medium, and suspension of the cells in mineral medium; (iii) after treatment of the cells with enzyme release buffer (50 mM potassium phosphate [pH 7]–10 mM 2-mercaptoethanol–10 mM dithiothreitol–10 mM $MgSO_4$), washing, and suspension of the cells in mineral medium. The reaction was started by the addition of glucose, fructose, or sucrose to a final concentration of 2 mM. Inulin-dependent oxygen consumption was assayed with 0.2% inulin. In the case of sucrose and inulin, the oxygen consumption rate increased with reaction time and became constant after 10 min. These final values, corrected for endogenous respiration, were used to calculate the rate of sugar-dependent oxygen consumption.

Fractionation of cultures for enzyme assays. Cells and culture supernatants were assayed for inulinase, invertase, and α-glucosidase activities. For the fractionation of cultures into three enzyme preparations, the method described by Rouwenhorst et al. (20) was used. Enzyme activity present in the culture fluid is referred to as supernatant enzyme. Enzyme released from the cell wall by incubation of the cells in enzyme release buffer and incubation for 1.5 h at 30°C is referred to as cell wall enzyme. The activity solubilized only by means of sonication is referred to as cell-bound enzyme.

Enzyme assays. Sucrose- and inulin-hydrolyzing activities were measured by following the rate of appearance of monosaccharides with the glucose/fructose Test Combination of Boehringer (Boehringer GmbH, Mannheim, Federal Republic of Germany) in the presence of 2% sucrose or 2% inulin in a 0.1 M sodium acetate buffer (pH 4.5) at 50°C.

α-Glucosidase activity was determined with *o*-nitrophenyl-α-D-glycopyranoside (Boehringer) as a substrate. Enzyme preparations were added to a prewarmed (33°C) solution of 0.1 M sodium phosphate (pH 7), 10 mM KCl, 10 mM 2-mercaptoethanol, 1 mM $MgSO_4$, and 4 mg of *o*-nitrophenyl-α-D-glycopyranoside per ml. The hydrolysis of *o*-nitrophenyl-α-D-glycopyranoside into 2-nitrophenol and D-glucose was followed on-line at 420 nm in a Vitalab 20 spectrophotometer (Vital Scientific, Dieren, The Netherlands).

Analytical methods. Biomass concentrations were measured by drying culture samples to constant weight at 70°C after membrane filtration (pore size, 0.45 μm; Schleicher & Schuell, Dassel, Federal Republic of Germany) and washing.

Nondenaturing polyacrylamide gel electrophoresis and detection of sucrose-hydrolyzing activity in the gels were done by the methods of Rouwenhorst et al. (19).

Chemicals. Fructose, glucose, sucrose, and 2-mercaptoethanol were purchased from Baker Chemicals BV, Deventer, The Netherlands. Dithiothreitol and inulin (chicory root) were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Sucrose- and inulin-dependent oxygen consumption by cell suspensions of *K. marxianus* var. *marxianus*. Sucrose and inulin can be hydrolyzed into the monosaccharides glucose and fructose. These monosaccharides are subsequently catabolized by the cells at the expense of oxygen. Thus, measurement of oxygen consumption after the addition of sucrose or inulin to yeast cells is a convenient way for the determination of in vivo invertase and inulinase activities. The cell walls of yeasts may act as a permeability barrier to the polymer inulin but not to the disaccharide sucrose (21). Therefore, it was of interest to investigate whether treatments that lead to removal of inulinase have the same effect on oxygen consumption with sucrose as with inulin. Washing cells results in removal of enzyme activity present in the culture fluid (i.e., supernatant enzyme). Incubation of the cells for 1.5 h at 30°C in potassium phosphate buffer (50 mM, pH 7) or in potassium phosphate buffer containing sulfhydryls (enzyme release buffer) and subsequent washing may give further depletion of enzyme activity because of the solubilization of part of the enzyme retained in the cell wall (20).

Washing or treatment with sulfhydryls of *K. marxianus* var. *marxianus* cells had no effect on fructose and glucose oxidation. The oxygen consumption rate after the addition of monosaccharide remained 7.3 ± 0.2 mmol h^{-1} g of cell (dry weight)$^{-1}$. The oxygen consumption rate of untreated *K. marxianus* var. *marxianus* cell suspensions with sucrose was equal to that observed with the monosaccharides glucose and fructose (Fig. 1). Washing of *K. marxianus* var. *marxianus* cells had no effect on the sucrose-dependent oxygen consumption rates, indicating that the inulinase activity present in the cell wall was high enough to saturate monosaccharide catabolism. Removal of part of the cell wall inulinase by incubation of the cells in either 50 mM potassium phosphate buffer (pH 7) or enzyme release buffer resulted in a decrease of sucrose-dependent oxygen consumption rates by 20 and 40%, respectively. Apparently, after these treatments hydrolysis had become the rate-limiting step in sucrose catabolism. By the addition of a proportional amount of culture supernatant, the oxygen consumption rate could be completely restored to the level observed with untreated cell suspension.

The oxygen consumption rate of untreated *K. marxianus* var. *marxianus* cell suspensions with inulin was about 8% lower than with sucrose or fructose (Fig. 1). The addition of

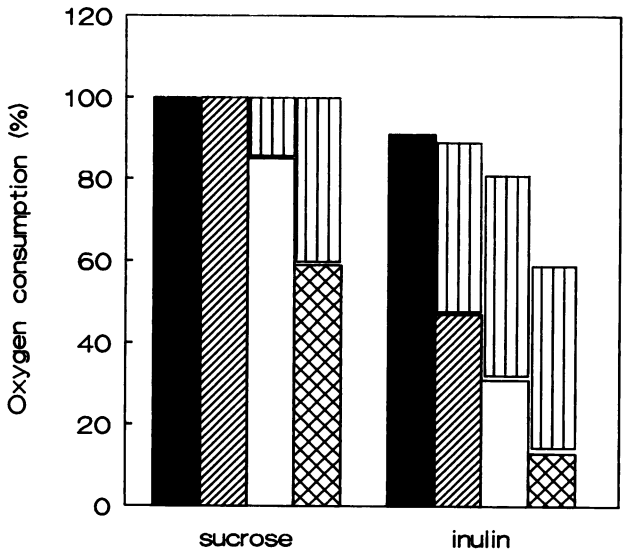

FIG. 1. Effect of inulinase removal on oxygen consumption by suspensions of *K. marxianus* var. *marxianus* CBS 6556. The oxygen consumption rates of fresh chemostat-cultivated cell suspension (■), washed cells (▨), cells treated with phosphate buffer (pH 7) (□), cells treated with enzyme release buffer (▩), and after addition of supernatant (▥) were determined after fourfold dilution with mineral medium and addition of 2 mM sucrose or 0.2% inulin. Cells were obtained from a steady-state chemostat culture grown on sucrose at pH 5.5 and 33°C. Oxygen consumption of 100% equals an oxygen consumption rate of 7.3 mmol h^{-1} g of cell (dry weight)$^{-1}$ as observed with glucose or fructose.

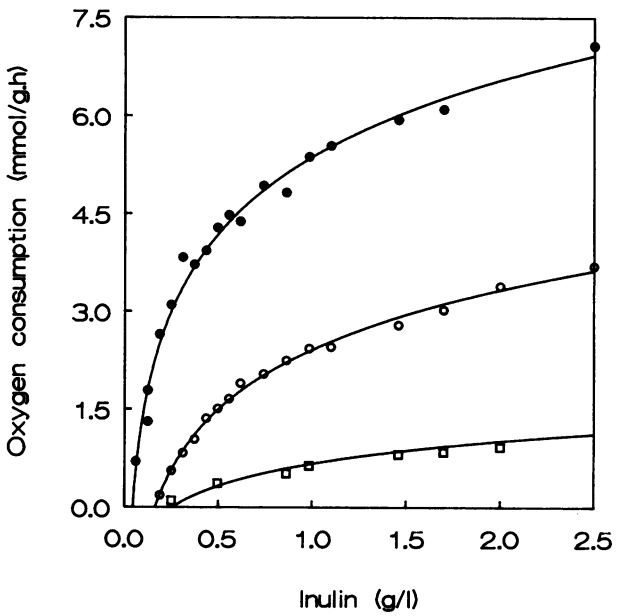

FIG. 2. Relationship between inulin concentration and oxygen consumption rates for fresh culture suspension (●), washed cells (○), and sulfhydryl-treated cells (□) of *K. marxianus* var. *marxianus* CBS 6556. Cells were obtained from a steady-state chemostat culture grown on sucrose at pH 5.5 and 33°C.

extra culture supernatant, which results in a higher inulinase concentration, increased the inulin-dependent oxygen consumption rate to that observed with sucrose or fructose. Apparently, in untreated cell suspensions, the amount of inulinase is just below the level required to saturate the fructose-oxidizing capacity of the cells.

The removal of supernatant inulinase by washing the cells with mineral medium led to a 50% decrease in the inulin-dependent oxygen consumption rate. An even greater effect was observed when the cells were suspended and incubated in potassium phosphate buffer or in enzyme release buffer. In these cases, the oxygen consumption rate decreased by 70 and 82%, respectively. The addition of culture supernatant to washed and sulfhydryl-treated cells resulted in an equal restoration of the oxygen consumption rates by approximately 50% of the original rates (Fig. 1). When excessive supernatant was added, the original rate of inulin-dependent oxygen consumption was fully restored (data not shown).

To obtain insight into the factor(s) that affects the inulin-hydrolyzing activity, the oxygen consumption rates of untreated, washed, and thiol-treated cells of *K. marxianus* var. *marxianus* were determined in relation to inulin concentration (Fig. 2). From the curves it is obvious that the oxygen consumption rate changed when cells were subjected to treatments that remove inulinase. A threshold level of inulin was required for the detection of inulin-dependent oxygen consumption. This threshold concentration increased when more inulinase was removed from the cells (Fig. 2). By extrapolation of Lineweaver-Burk plots, derived from the data in Fig. 2, and disregarding very low oxygen consumption rates, K_s and $V_{max\ O_2}$ values could be calculated. The untreated cell suspension of *K. marxianus* var. *marxianus* gave the highest $V_{max\ O_2}$ value and the lowest K_s value (7.1 mmol of O_2 h^{-1} g of cell [dry weight]$^{-1}$ and 0.3 g of inulin liter^{-1}, respectively). The removal of inulinase present in the culture fluid by washing with mineral medium resulted in an overall decrease in the oxygen consumption rate (maximum, 5.6 mmol of O_2 h^{-1} g of cell [dry weight]$^{-1}$) and an increase in the K_s value (1.3 g of inulin liter^{-1}). A similar effect was observed when cells were treated with enzyme release buffer in that the maximal oxygen consumption rate and K_s became 2.2 mmol of O_2 h^{-1} g of cell (dry weight)$^{-1}$ and 2.5 g of inulin liter^{-1}, respectively. By removing inulinase from the system, hydrolysis of inulin became rate limiting and thus oxygen consumption rates decreased. The increase in the affinity constant of whole cells towards inulin might be, at least in part, a result of a decrease in accessibility of the inulinase to the inulin molecules.

Sucrose- and inulin-dependent oxygen consumption in cell suspensions of *K. marxianus* var. *drosophilarum*. The yeast *K. marxianus* var. *drosophilarum* was originally included in our study as a representative of a sucrose-utilizing *Kluyveromyces* strain that is unable to grow on inulin. It was anticipated that growth of this yeast on sucrose would involve a genuine invertase, i.e., one not capable of inulin hydrolysis.

K. marxianus var. *drosophilarum* was grown in a sucrose-limited chemostat culture at pH 5.5 and 33°C. Cells from these cultures showed a sucrose-dependent oxygen consumption rate that was not influenced by washing or by treatment with enzyme release buffer (Fig. 3A). The oxygen consumption rate remained constant and was equal to the oxygen consumption rate with glucose or fructose (4.2 mmol of O_2 h^{-1} g of cell [dry weight]$^{-1}$). As expected, no oxygen consumption was observed when untreated *K. marxianus* var. *drosophilarum* cells were given inulin as substrate. Surprisingly, however, washing of these cells resulted in an inulin-dependent oxygen consumption. A further increase of the oxygen consumption rate was observed when the cells were treated with enzyme release buffer (Fig. 3A).

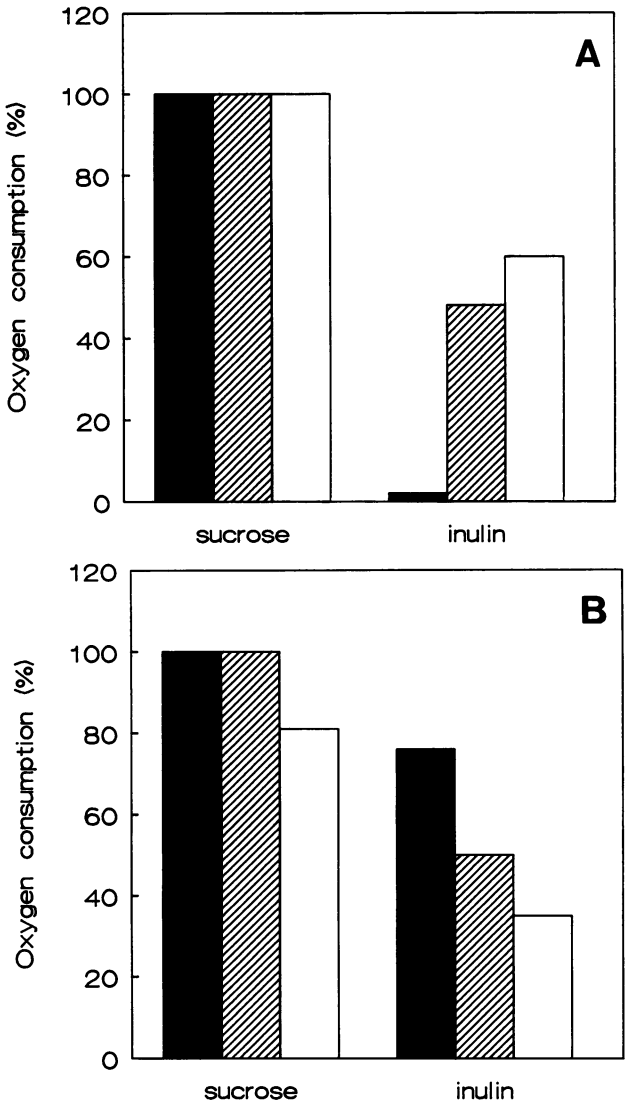

FIG. 3. Effect of enzyme depletion on oxygen consumption rate of *K. marxianus* var. *drosophilarum* CBS 2103. The oxygen consumption rates of cell suspension (■), washed cells (▨), and cells treated with enzyme release buffer (□) were determined after fourfold dilution with mineral medium and addition of 2 mM sucrose or 0.2% inulin. Cells were obtained from a steady-state chemostat culture grown on sucrose at pH 5.5 and 30°C (A) or from a steady-state chemostat culture grown on sucrose at pH 4.5 and 33°C (B).

These results indicate that *K. marxianus* var. *drosophilarum* contains a cryptic inulinase activity that becomes functional after washing or treatment of the cells with sulfhydryls. Indeed, enzyme assays showed that, in contrast to the situation in *K. marxianus* var. *marxianus*, culture supernatants of *K. marxianus* var. *drosophilarum* did not contain sucrose- or inulin-hydrolyzing activity. Apparently, the inulinase in this strain is strictly cell wall bound and only becomes accessible to inulin after special treatment of the cells. The localization of inulinase in this strain was strongly affected by the culture conditions, i.e., when *K. marxianus* var. *drosophilarum* was grown on sucrose at pH 4.5 instead of at pH 5.5, untreated cell suspensions of *K. marxianus* var. *drosophilarum* were able to utilize not only sucrose but also inulin. Results similar to those observed for *K. marxianus* var. *marxianus* were now obtained (Fig. 3B). Supernatants of cultures grown at pH 4.5 contained sucrose- and inulin-hydrolyzing activities with an S/I ratio (relative activity with sucrose and inulin) of 22.

TABLE 1. Aerobic and anaerobic utilization of some sugars by *Kluyveromyces* species[a]

Strain[b]	Utilization of substrate[c]			
	Sucrose	Raffinose	Maltose	Inulin
K. marxianus var. *drosophilarum* CBS 2103	+	+	−	−
K. marxianus var. *lactis*				
CBS 683	+	+	+k	−
CBS 739	+	+	+k	−
CBS 1067	+	+	+k	−
CBS 2359	+	+	+k	−
CBS 8043	+k	−	+k	−
K. marxianus var. *marxianus*				
CBS 397	+	+	−	+
CBS 6556	+	+	−	+
K. marxianus var. *vanudenii* CBS 5669	+	+k	−	−
K. lodderi CBS 2758	+	+	−	−
K. waltii CBS 6430	+	+	−	−
S. cerevisiae CBS 8066	+	+	+	−
S. kluyveri UCD 51-242	+	+	+k	−

[a] The taxonomic tests of the Yeast Division of the Centraal bureau voor Schimmelcultures, Delft, The Netherlands, were used. Sugar utilization was tested at pH 5.6 and 25°C.
[b] Nomenclature according to van der Walt and Johanssen (28).
[c] +, Growth; −, no growth; k, Kluyver effect with oligosaccharide.

Sucrose and inulin utilization by *Kluyveromyces* and *Saccharomyces* species. The rather unexpected presence of an inulinase in *K. marxianus* var. *drosophilarum*, the localization of which is dependent on cultivation conditions, led us to reinvestigate the ability of this and other *Kluyveromyces* strains to assimilate inulin. *S. cerevisiae* was included as the reference organism, since the inability of this yeast to utilize inulin is well established (22). The yeast *S. kluyveri* UCD 51-242 was included since it has been reported to contain an invertase, although no homology was found between its genome and the *SUC2* gene of *S. cerevisiae* (8).

In yeast systematics, physiological properties such as the aerobic and anaerobic utilization of saccharides are used to describe and identify yeasts. These physiological characteristics are often dependent on the culture conditions employed. Reproducibility of the taxonomic tests has been achieved by international standardization. Aerobic utilization of saccharides is generally tested in reagent tubes with 5 ml of medium containing Yeast Nitrogen Base (pH 5.6) supplemented with 50 mM of sugar. These tubes are then incubated with gentle shaking at 25°C for 3 to 21 days. Fermentative utilization is tested in Durham tubes with the same medium and incubation at 25°C for 7 days (6, 28).

The growth characteristics of 13 *Kluyveromyces* and *Saccharomyces* strains with the oligosaccharides maltose, sucrose, and raffinose and with the polysaccharide inulin are listed in Table 1. All of these yeasts are able to assimilate sucrose aerobically. The nature of the sucrose-hydrolyzing enzyme in the various strains can be predicted when growth on other saccharides is taken into account (4, 5). Growth on

TABLE 2. Growth of yeast species on mineral medium with 2% (wt/vol) inulin at different pH values and temperatures in batch cultures

Strain	Growth[a] at:								
	pH 4.5			pH 5.2–5.5			pH 6.0–6.5		
	25°C	30°C	35°C	25°C	30°C	35°C	25°C	30°C	35°C
K. marxianus var. *drosophilarum* CBS 2103	+	+	++	v	v	+	−	−	+
K. marxianus var. *vanudenii* CBS 5669	−	+	+	v	v	−	−	−	−
S. kluyveri UCD 51-242	+	+	++	−	−	−	−	−	−

[a] ++, Growth within 2 days; +, growth within 4 days; ±, growth within 8 days; −, no growth; v, variable growth.

both sucrose and inulin indicates the production of an inulinase (i.e., *K. marxianus* var. *marxianus* CBS 6397 and CBS 6556). Yeast strains that are able to utilize raffinose but not inulin possess an extracellular invertase (i.e., *K. marxianus* var. *drosophilarum* CBS 2103; *K. marxianus* var. vanudenii CBS 5669; *K. lodderi* CBS 2758; *K. waltii* CBS 6430; *K. marxianus* var. *lactis* CBS 683, CBS 739, CBS 1067, and CBS 2359; *S. cerevisiae* CBS 8066; and *S. kluyveri* UCD 51-242) (6).

An important feature of oligosaccharide utilization is the occurrence of the Kluyver effect. This effect is defined as the inability of a facultatively fermentative yeast to utilize a certain oligosaccharide anaerobically, although it readily utilizes this sugar aerobically (4). The Kluyver effect only occurs with oligosaccharides that are hydrolyzed intracellularly. The effect is probably caused by the inability of the yeast to transport the oligosaccharides across the plasma membrane under anaerobic conditions (4, 5). Yeasts that show the Kluyver effect with both sucrose and maltose are supposed to metabolize the sucrose only via an intracellular α-glucosidase (i.e., *K. marxianus* var. *lactis* CBS 8043). The coincidence of the occurrence of the Kluyver effect with both maltose (an α-glucoside) and sucrose in certain yeast species is not surprising, since sucrose can be regarded both as an α-glucoside and as a β-fructoside.

In yeast systematics, utilization of carbon sources is tested under standard conditions, i.e., with Yeast Nitrogen Base (pH 5.6) at 25°C in reagent tubes that are slowly agitated. Growth conditions different from those used in yeast systematics did not result in changes in growth characteristics with disaccharides of most of the yeasts listed in Table 1. However, 3 of 13 yeast strains behaved anomalously (Table 2). The use of shake flasks instead of tubes, a synthetic mineral medium instead of Yeast Nitrogen Base, and increased growth temperature changed the growth characteristics of *K. marxianus* var. *drosophilarum* CBS 2103 and of *K. marxianus* var. *vanudenii* CBS 5669. When, moreover, the culture pH was changed to pH 4.5, these two *Kluyveromyces* strains mentioned and *S. kluyveri* UCD 51-242 were capable of fast utilization of inulin. The use of higher pH values of 6.0 to 6.5 did not result in fast growth on inulin. Weak growth on inulin of the *Kluyveromyces* species at pH 5.5 was observed only at the highest temperature tested (35°C). Apparently, utilization of inulin by some yeasts is primarily dependent on culture pH and to a lesser extent on the incubation temperature. The possible effects of aeration, which is much better in shake flasks than in the shake tubes used in yeast taxonomy, were not investigated.

The above results explain the absence of in vivo inulinase activity in *K. marxianus* var. *drosophilarum* pregrown at pH 5.5 on sucrose. When grown at this pH, the inulinase is retained in the cell wall and is not accessible to inulin, unless the cells are washed or treated with thiols. When grown at pH 4.5, the inulinase is partially secreted into the culture liquid, as in *K. marxianus* var. *marxianus*. In this yeast the localization of inulinase is independent of culture pH (20). The two other yeast species that showed growth on inulin only at the lower pH value, *K. marxianus* var. *vanudenii* and *S. kluyveri*, were also tested for sucrose- and inulin-dependent oxygen consumption. Oxygen consumption patterns after various treatments were similar to those found with *K. marxianus* var. *drosophilarum* when these yeasts were grown in a chemostat culture on sucrose at pH 4.5 instead of at pH 5.5 (data not shown).

Sucrose-dependent oxygen consumption by yeasts that do not contain an inulinase. Treatment of *S. cerevisiae* cells with sulfhydryls has little effect on the solubilization of invertase (10, 29). Indeed, the sucrose-dependent oxygen consumption rate in *S. cerevisiae* was not affected by either washing of the cells or treatment of the cells with sulfhydryls. Oxygen consumption rates with sucrose were the same as those observed with glucose or fructose (results not shown). Similar observations were made with seven invertase-producing *K. marxianus* strains of the varieties *lactis*, *lodderi*, and *waltii*, with the exception of *K. marxianus* var. *lactis* CBS 739 (Table 1). This last yeast showed a much lower oxygen consumption rate with fructose (60%) than with glucose or sucrose. As mentioned above, *K. marxianus* var. *lactis* CBS 8043 probably does not produce an inulinase or an invertase. Hydrolysis of sucrose in this organism may occur via an intracellular α-glucosidase. Irrespective of treatment of the cells, this yeast showed a sucrose-dependent oxygen consumption rate that was 70% of the rate observed with glucose. Obviously, monosaccharide catabolism is not saturated when sucrose is the substrate. The rate-limiting process in this case may be either transport of sucrose into the cell or hydrolysis of sucrose by internal α-glucosidase. None of the *K. marxianus* strains of the varieties *lactis*, *lodderi*, and *waltii* listed in Table 1 showed inulin-dependent oxygen consumption either as untreated cell suspension or after washing with thiols. This is in line with their inability to grow on inulin under a variety of culture conditions.

Characterization of enzyme activities. Snyder and Phaff (22) introduced the S/I ratio, the ratio of the activities with sucrose and inulin as substrates, to discriminate between invertases and inulinases. Inulinases are characterized by S/I ratios lower than 50 (27). In cell extracts of chemostat-grown yeasts, hydrolase activities with sucrose, maltose, and inulin were determined. The yeasts *K. marxianus* var. *drosophilarum*, *K. marxianus* var. *vanudenii*, and *S. kluyveri* showed S/I ratios of 22, 9, and 18, respectively. These S/I ratios are comparable to the value of 15 found for the *K. marxianus* var. *marxianus* inulinase (20). The enzyme assays confirmed that the yeast strains that did not produce an inulinase contained an invertase (S/I ratios > 1,200) or an α-glucosidase, or both, as was predicted by the growth characteristics (Table 1) and sucrose-dependent oxygen consumption patterns. The strain that showed the Kluyver effect with both sucrose and maltose, *K. marxianus* var. *lactis* CBS 8043, probably possessed an α-glucosidase. The sucrose-hydrolyzing activity in cell extracts of this strain exhibited optimal activity at pH 7, whereas under optimal conditions for invertase (pH 5) activity was greatly reduced.

Cell extracts of *K. marxianus* var. *marxianus* CBS 6556, *S. cerevisiae*, *K. marxianus* var. *lactis* CBS 739, *K. marx-*

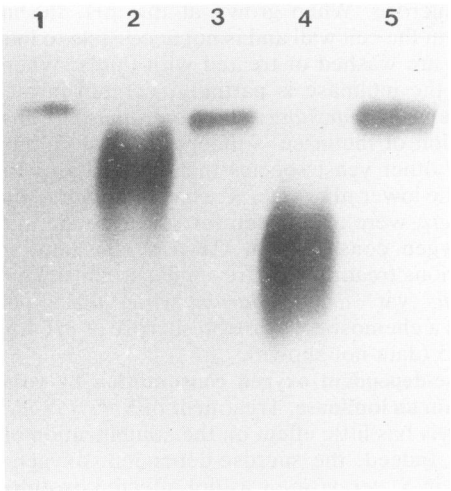

FIG. 4. Sucrose-hydrolyzing activities after nondenaturing polyacrylamide gel electrophoresis of invertases and inulinases in cell extracts of different yeast species. Invertase of *K. waltii* CBS 6430 (lane 1), *K. marxianus* var. *lactis* CBS 739 (lane 3), and *S. cerevisiae* CBS 8066 (lane 5), and inulinase of *K. marxianus* var. *drosophilarum* CBS 2103 (lane 2) and of *K. marxianus* var. *marxianus* CBS 6556 (lane 4) were applied on a 7% polyacrylamide gel, and the gel was stained for sucrose-hydrolyzing activity.

ianus var. *drosophilarum*, and *K. waltii* CBS 6430 were subjected to nondenaturing polyacrylamide gel electrophoresis and the gel was stained for sucrose-hydrolyzing activity. The invertases of *K. waltii*, *K. marxianus* var. *lactis*, and *S. cerevisiae* (Fig. 4, lanes 1, 3, and 5) showed similar migration distances. The inulinases of *K. marxianus* var. *drosophilarum* and *K. marxianus* var. *marxianus* (Fig. 4, lanes 2 and 4) showed lower activity bands. However, these two inulinases did not migrate in the same way. The sucrose activity band of the inulinase of *K. marxianus* var. *drosophilarum* migrated between those of the *K. marxianus* var. *marxianus* inulinase (molecular mass, 400 kDa) and of the *S. cerevisiae* invertase (molecular mass, 800 kDa).

DISCUSSION

Occurrence of inulin utilization among yeasts. According to systematic studies, *K. marxianus* var. *marxianus*, which includes the former species *Kluyveromyces fragilis* and *Kluyveromyces bulgaricus*, is the only representative of the genus capable of inulin utilization (6, 28). However, it appears that utilization of inulin is much more widespread among *Kluyveromyces* species (Table 2). It may well be that also other yeast genera in which inulin utilization has been established (e.g., the genera *Lipomyces*, *Hansenula*, and *Candida*) may contain more species capable of inulin utilization than those recognized until now.

Especially interesting was the finding that the yeast *S. kluyveri* UCD 51-242 could grow on inulin when culture conditions differed from those generally employed in yeast systematics (Table 2). Carlson and Botstein (8) reported that this yeast produced an invertase different from that of *S. cerevisiae*. These authors investigated the physical structure of the invertase-coding (*SUC*) gene family by using cloned *SUC2* DNA probes to detect homologous sequences in 14 *S. cerevisiae* strains and in 10 closely related *Saccharomyces* species. They found that the *SUC* DNA sequence was highly conserved within the genus *Saccharomyces*. Only DNA from *S. kluyveri* UCD 51-242 failed to hybridize with the *SUC2* DNA probe. Carlson and Botstein (8) concluded that this species was more distantly related to *S. cerevisiae* than the other species tested and that during evolution its *SUC* gene had diverged sufficiently to leave no detectable homology. The growth of *S. kluyveri* on inulin, reported in this paper, and the S/I ratio of the enzyme indicates that this organism does not produce an invertase but an inulinase, which is a completely different enzyme (19).

The unrecognized ability of some yeasts to grow on inulin is mainly a result of the fact that in yeast systematics, for reasons of standardization and convenience, growth on sugars is tested under special cultivation conditions (4, 6, 28). Since the utilization of inulin strongly depends on growth conditions in some strains (Tables 1 and 2), care should be taken in interpreting the growth characteristics derived from taxonomic tests. It is conceivable that growth not only on inulin but also on polymers in general is strongly affected by growth conditions.

Localization of inulinase and invertase in yeasts. The localization of inulinase and invertase in *Kluyveromyces* and *Saccharomyces* strains depends on the yeast strain and on the cultivation conditions (5, 25). In *S. cerevisiae*, almost all invertase produced is retained in the cell wall (10). A large part of the inulinase of *K. marxianus* var. *marxianus* is secreted into the culture fluid (16, 20). Removal of the supernatant enzyme of *K. marxianus* var. *marxianus* had no effect on sucrose consumption, but treatment of the cells with sulfhydryls decreased the sucrose-dependent oxygen consumption rate and thus limited sucrose catabolism (Fig. 1). Contrary to invertase, inulinase is able to hydrolyze fructans like inulin and levan (22, 23). These polysaccharides do not enter the cell wall of yeasts (21) and hydrolysis must occur outside the cell wall. Measurements of inulin-dependent oxygen consumption of *K. marxianus* var. *marxianus* cells revealed that both inulin concentration and inulinase localization determine the rate of inulin hydrolysis (Fig. 1 and 2).

Cultivation of *K. marxianus* var. *drosophilarum*, *K. marxianus* var. *vanudenii*, and *S. kluyveri* at lower pH values and at higher growth temperatures than those normally used in yeast systematics allowed growth of these strains on inulin (Table 2). During growth in sucrose-limited continuous cultures at pH 5.5, a pH value that does not support growth on inulin, these yeasts produced an inulinase that is located in the cell wall. Cell suspensions were not able to utilize inulin, but washing or treatment with sulfhydryls restored this ability (Fig. 3A). Obviously, these cells acquired their ability to hydrolyze inulin as a result of the removal of a permeability barrier in the outer regions of the cell wall. This enables either inulin to enter the cell wall or inulinase to diffuse out of the cell wall. Washing of yeast cells has been reported to affect the lipid, protein, and carbohydrate contents of the yeast cell wall (25). As a result of this treatment, the carbohydrate content increases whereas the protein and lipid contents decrease. The decrease in lipid content could be of special importance, since it has been suggested that the lipid plays a role in maintaining the ordered structure of the wall (25). The treatment of yeast cells with sulfhydryls like β-mercaptoethanol and dithiothreitol increases the permeability of the cell wall by reducing the disulfide linkages in the outer layers of the cell wall, thus giving rise to the occurrence of pores (9, 14).

The alteration of growth conditions promoted secretion of inulinase into the culture liquid by cells of *K. marxianus* var. *drosophilarum*, *K. marxianus* var. *vanudenii*, and *S.*

kluyveri. Apparently, molecular sieving by the cell wall was less efficient than during cultivation conditions employed in yeast systematics. Unfortunately, reports available on pH- and temperature-induced morphological changes in yeast cell walls are limited. The available information mainly concerns the chemical composition of cell walls but not their structure (13, 25). The inability of yeast cells to grow on inulin in spite of the presence of an inulinase has also been reported for a respiratory-deficient mutant of *K. marxianus* var. *marxianus* by Guiraud et al. (12). These authors observed that the mutant could still grow on sucrose but that the ability to grow on inulin was lost. Hydrolysis of sucrose appeared to be catalyzed by inulinase that, as a side effect of the mutation, was completely retained in the cell wall.

The reason for the difference between *S. cerevisiae* and *K. marxianus* with respect to the retention of their hydrolyzing enzymes in the cell walls remains unclear. Both the molecular mass of the native enzyme (invertase is a much larger aggregate than inulinase; Fig. 4) and the cell wall composition may be of importance. Compared with the cell wall of *S. cerevisiae*, the wall of *K. marxianus* var. *marxianus* is much more sensitive to changes in ionic strength of the surrounding medium (29), changes in pH (9, 29), and treatment with thiols (9, 14, 29). The latter may indicate that the cell wall of *K. marxianus* var. *marxianus* is less rigid than that of *S. cerevisiae*.

The physiological role of inulinase. In addition to structural differences and differences in enzyme kinetics (10, 11, 20, 22, 27), invertase and inulinase also differ in physiological function. The physiological role of invertase mainly concerns the hydrolysis of sucrose within the cell wall. The main physiological role of inulinase is the breakdown of fructans outside the cell wall. Retention of invertase within the cell wall results in an enzyme concentration and a possible way to outcompete non-invertase-producing microorganisms (1, 4). That excretion of invertase into the culture liquid may be regarded as disadvantageous is exemplified in bakers' yeast production. The small amounts of the invertase released by this yeast are sufficient to promote growth of infections like *Candida kruseii* (24), a yeast unable to grow on sucrose. On the other hand, with inulin as a substrate, excretion of inulinase into the culture medium is a necessity, since this polymer cannot penetrate the cell wall. The cell wall-retained inulinase does contribute to inulin metabolism but probably only when small oligofructosides are present.

In *K. marxianus* var. *drosophilarum* and *K. marxianus* var. *vanudenii*, the localization of inulinase strongly depended on environmental pH. Only at low pH values were these organisms able to consume inulin. If this pH-dependent localization of inulinase also exists under natural conditions, it follows that inulin degradation by these yeasts is carried out only in acidic environments. In this respect it is relevant that these yeasts can be found in root exudates of all sorts of trees and in the alimentary tracts of fruit flies (6, 17). These environments are acidic and therefore may allow inulinase to become functional.

Applied aspects of inulinase-producing yeasts. The observed decrease in inulin-dependent oxygen consumption caused by washing the cells could be of importance in possible applications of *K. marxianus* var. *marxianus*. The production of D-fructose or ethanol from inulin by immobilized *K. marxianus* in a continuous process is in most cases lower than expected (3, 26). This might result from a limited inulin-metabolizing capacity caused by low inulinase concentrations, i.e., cells are washed before immobilization, and high flow rates are used during the process. Both process steps may lead to loss of inulinase activity.

Studies of the localization of extracellular enzymes could be of interest for the expression of heterologous genes in yeasts. The downstream processing of heterologous gene products depends especially on their localization. By adapting cultivation conditions, all of the protein may be retained in the cell wall. Subsequently, it can then be easily released by changing culture conditions or by chemical treatment of the cells without a significant loss of cell viability. This provides the opportunity to concentrate the desired protein without cell disruption and may allow recycling of the cells.

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LITERATURE CITED

1. **Arnold, W. N.** 1981. Enzymes, p. 1–46. *In* W. N. Arnold (ed.), Yeast cell envelopes: biochemistry, biophysics and ultrastructure, vol. 2. CRC Press, Inc., Boca Raton, Fla.
2. **Arnold, W. N.** 1987. Hydrolytic enzymes, p. 369–391. *In* D. R. Berry, I. Russell, and G. G. Stewart (ed.), Yeast biotechnology, 2nd ed. Allen & Unwin, London.
3. **Bajpai, P., and A. Margaritis.** 1986. Continuous ethanol production from Jerusalem artichoke stalks using immobilized cells of *Kluyveromyces marxianus*. Process Biochem. **21**:86–89.
4. **Barnett, J. A.** 1977. The nutritional tests in yeast systematics. J. Gen. Microbiol. **99**:183–190.
5. **Barnett, J. A.** 1981. The utilization of disaccharides and some other sugars by yeasts. Adv. Carbohydr. Chem. Biochem. **39**:347–404.
6. **Barnett, J. A., R. W. Payne, and D. Yarrow.** 1983. Yeasts: characteristics and identification. Cambridge University Press, Cambridge.
7. **Bruinenberg, P. M., J. P. van Dijken, and W. A. Scheffers.** 1983. An enzymic analysis of NADPH production and consumption in *Candida utilis*. J. Gen. Microbiol. **129**:965–971.
8. **Carlson, M., and D. Botstein.** 1983. Organization of the *SUC* gene family in *Saccharomyces*. Mol. Cell. Biol. **3**:351–359.
9. **Davies, R., and F. J. Wayman.** 1975. The effect of thiols on *Saccharomyces fragilis*. Antonie van Leeuwenhoek J. Microbiol. Serol. **41**:33–58.
10. **Esmon, P. C., B. E. Esmon, I. E. Schauer, A. Taylor, and R. Schekman.** 1987. Structure, assembly, and secretion of octameric invertase. J. Biol. Chem. **262**:4387–4394.
11. **GrootWassink, J. W. D., and S. E. Fleming.** 1980. Non-specific β-fructofuranosidase (inulase) from *Kluyveromyces fragilis*: batch and continuous fermentation, simple recovery method and some industrial properties. Enzyme Microb. Technol. **2**:45–53.
12. **Guiraud, J. P., D. Mouillet, J. Bourgi, M. Claisse, and P. Galzy.** 1988. Impairment of inulin assimilation and β-fructosidase activity due to a petite mutation in *Kluyveromyces marxianus*. Antonie van Leeuwenhoek J. Microbiol. Serol. **54**:127–138.
13. **Hunter, K., and A. H. Rose.** 1972. Influence of growth temperature on the composition and physiology of micro-organisms. J. Appl. Chem. Biotechnol. **22**:527–540.
14. **Kidby, D. K., and R. Davies.** 1970. Thiol induced release of invertase from cell walls of *Saccharomyces fragilis*. Biochim. Biophys. Acta **201**:261–266.
15. **Kovaljova, N. S., and V. V. Yurkevich.** 1978. On the existence of β-fructosidases of inulinase type in yeast, p. S VI 3. *In* P. Galzy, A. Arnaud, C. Bizeau, and G. Moulin (ed.), Proceedings of the VIth International Specialized Symposium on Yeasts: Metabo-

lism and regulation of cellular processes. ENSAM-CRAM, Montpellier, France.
16. **Lam, K. S., and J. W. D. GrootWassink.** 1985. Efficient, non-killing extraction of β-D-fructofuranosidase (an exo-inulase) from *Kluyveromyces fragilis* at high cell-density. Enzyme Microb. Technol. **7**:239–242.
17. **Phaff, H. J., and W. T. Starmer.** 1987. Yeasts associated with plants, insects, and soil, p. 123–180. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, 2nd ed., vol. 1. Academic Press, Inc. (London), Ltd., London.
18. **Phelps, C. F.** 1965. The physical properties of inulin solutions. Biochem. J. **95**:41–47.
19. **Rouwenhorst, R. J., M. Hensing, J. Verbakel, W. A. Scheffers, and J. P. van Dijken.** 1990. Structure and properties of the extracellular inulinase of *Kluyveromyces marxianus* CBS 6556. Appl. Environ. Microbiol. **56**:3337–3345.
20. **Rouwenhorst, R. J., L. E. Visser, A. A. van der Baan, W. A. Scheffers, and J. P. van Dijken.** 1988. Production, distribution, and kinetic properties of inulinase in continuous cultures of *Kluyveromyces marxianus* CBS 6556. Appl. Environ. Microbiol. **54**:1131–1137.
21. **Scherrer, R., L. Louden, and P. Gerhardt.** 1974. Porosity of the yeast cell wall and membrane. J. Bacteriol. **118**:534–540.
22. **Snyder, H. E., and H. J. Phaff.** 1960. Studies on a beta-fructosidase (inulinase) produced by *Saccharomyces fragilis*. Antonie van Leeuwenhoek J. Microbiol. Serol. **26**:433–452.
23. **Snyder, H. E., and H. J. Phaff.** 1962. The pattern of action of inulinase from *Saccharomyces fragilis* on inulin. J. Biol. Chem. **237**:2438–2441.
24. **Suihko, M.-L., and V. Mäkinen.** 1978. *Candida krusei* in the baker's yeast cultivation, p. SI 6. *In* P. Galzy, A. Arnaud, C. Bizeau, and G. Moulin (ed.), Proceedings of the VIth International Specialized Symposium on Yeasts: Metabolism and regulation of cellular processes. ENSAM-CRAM, Montpellier, France.
25. **Suomalainen, H., T. Nurminen, and E. Oura.** 1973. Aspects of cytology and metabolism of yeast, p. 109–167. *In* D. J. D. Hockenhull (ed.), Progress in industrial microbiology, vol. 12. Churchill Livingstone, Ltd., Edinburgh.
26. **Thonart, P., and C. Artois.** 1986. Inulin hydrolysis by an immobilized yeast cell reactor. Biotechnol. Bioeng. Symp. (1985) **15**:409–418.
27. **Vandamme, E. J., and D. G. Derycke.** 1983. Microbial inulinases: fermentation process, properties, and applications. Adv. Appl. Microbiol. **29**:139–176.
28. **van der Walt, J. P., and E. Johanssen.** 1984. Genus 13. *Kluyveromyces* van der Walt emend. van der Walt, p. 224–251. *In* N. J. W. Kreger-van Rij (ed.), The yeasts a taxonomic study, 3rd ed. Elsevier/North Holland Publishing Co., Amsterdam.
29. **Weimberg, R., and W. L. Orton.** 1966. Elution of exocellular enzymes from *Saccharomyces fragilis* and *Saccharomyces cerevisiae*. J. Bacteriol. **91**:1–13.