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Production, Distribution, and Kinetic Properties of Inulinase in Continuous Cultures of *Kluyveromyces marxianus* CBS 6556

ROBERT J. ROUWENHORST,* LEO E. VISSER, ADRIAAN A. VAN DER BAAN, W. ALEXANDER SCHEFFERS, AND JOHANNES P. VAN DIJKEN

Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

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From a screening of several *Kluyveromyces* strains, the yeast *Kluyveromyces marxianus* CBS 6556 was selected for a study of the parameters relevant to the commercial production of inulinase (EC 3.2.1.7). This yeast exhibited superior properties with respect to growth at elevated temperatures (40 to 45°C), substrate specificity, and inulinase production. In sucrose-limited chemostat cultures growing on mineral medium, the amount of enzyme decreased from 52 U mg of cell dry weight^{-1} at $D = 0.1$ h^{-1} to 2 U mg of cell dry weight^{-1} at $D = 0.8$ h^{-1}. Experiments with nitrogen-limited cultures further confirmed that synthesis of the enzyme is negatively controlled by the residual sugar concentration in the culture. High enzyme activities were observed during growth on nonsugar substrates, indicating that synthesis of the enzyme is a result of a derepression/repression mechanism. A substantial part of the inulinase produced by *K. marxianus* was associated with the cell wall. The enzyme could be released from the cell wall via a simple chemical treatment of cells. Results are presented on the effect of cultivation conditions on the distribution of the enzyme. Inulinase was active with sucrose, raffinose, stachyose, and inulin as substrates and exhibited an S/I ratio (relative activities with sucrose and inulin) of 15 under standard assay conditions. The enzyme activity decreased with increasing chain length of the substrate.

Representatives of the genus *Kluyveromyces* are well known for their ability to grow on fructans such as inulin. Inulin is a storage polysaccharide of plant origin and consists of a linear chain of β(2,1)-linked D-fructofuranose molecules terminated at the reducing end by a D-glucose residue. In yeasts, the enzyme responsible for the degradation of inulin is a nonspecific β-fructosidase (inulinase:2,1-D-fructanfructanohydrolase; EC 3.2.1.7) that liberates fructose molecules from sugars with β(2,1)-linked fructose units at the terminal, nonreducing end (6, 19). In spite of similarity in enzyme action and correspondence in affinity for sucrose, yeast inulinase is distinguished from another well-known β-fructosidase, invertase (β-D-fructofuranoside fructohydrolase; EC 3.2.1.26). The latter enzyme shows a low activity with higher-molecular-weight substrates such as inulin. The so-called S/I ratio (relative activities with sucrose and inulin) is now commonly used to discriminate between inulinase and invertase (24). This parameter, however, is strongly dependent upon the method used to determine enzyme activities (18, 26).

The inulinase of yeasts is an extracellular enzyme partially associated with the cell wall and partially excreted into the culture fluid. Regulation of inulinase synthesis in yeasts has been studied in batch and continuous cultures, mainly with complex media (1, 9, 16, 18). From these studies, it was concluded that the enzyme is inducible and subject to catabolite repression. Highest enzyme production so far was obtained with constitutive, derepressed mutants of *Kluyveromyces fragilis* in chemostat cultures (9, 11).

In our studies on the optimization of inulinase production by yeasts, *Kluyveromyces marxianus* CBS 6556 was found to exhibit many properties which compare favorably with those reported for other *Kluyveromyces* strains. These include fast growth on a wide range of substrates at temperatures above 40°C and high enzyme productivity. We present the results of a continuous-culture study on inulinase production by this strain.

MATERIALS AND METHODS

Microorganism and culture conditions. *K. marxianus* var. *marxianus* CBS 6556 was obtained from the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands, and maintained on YEPD agar slopes. YEPD contained the following, per liter of distilled water: yeast extract (Difco Laboratories, Detroit, Mich.), 10 g; Bacto-Peptone (Difco), 10 g; glucose, 20 g. The organism was grown at 40°C in a laboratory fermentor (Applikon, Schiedam, The Netherlands) with a working volume of 1 liter. Dissolved oxygen was measured with a polarographic oxygen electrode (Ingold, Urdorf, Switzerland) and controlled at 50 to 70% of air saturation. The pH was maintained at pH 4.5 by automatic addition of 1 M KOH or 0.5 M H_2SO_4.

For carbon- and energy-limited growth, mineral medium contained the following, per liter: $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; EDTA, 15 mg; $ZnSO_4 \cdot 7H_2O$, 0.45 mg; $FeSO_4 \cdot 7H_2O$, 3 mg, $CuSO_4 \cdot 5H_2O$, 0.3 mg, $CaCl_2 \cdot 2H_2O$, 0.45 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; $CoCl_2 \cdot 6H_2O$, 0.3 mg; $NaMoO_4 \cdot 2H_2O$, 0.04 mg; H_3BO_3, 1 mg; KI, 0.1 mg; silicon antifoaming agent (BDH Chemicals, Poole, Dorset, England), 0.025 ml; calcium pantothenate, 1 mg; nicotinic acid, 1 mg. The medium was sterilized at 120°C. For nitrogen-limited growth, the concentration of $(NH_4)_2SO_4$ was lowered to 200 mg liter^{-1}. Carbon sources (glucose, fructose, lactose, sucrose, inulin, and glycerol) were heat sterilized separately at 110°C, except for ethanol and fructose, which were filter sterilized. Carbon sources were added to give final concentrations of 2.5 g liter^{-1}, unless mentioned otherwise.

Fractionation of cultures for inulinase assays. Both cells

* Corresponding author.

and culture supernatants were routinely assayed for inulinase activity. The fraction of cell-associated enzyme that could be released by treatment with sulfhydryl reagents will be referred to as cell wall enzyme. The activity that could only be solubilized by means of sonication will be referred to as cell-bound enzyme. The fractionation of cultures into supernatant enzyme, cell wall enzyme, and cell-bound enzyme is described below.

(i) Supernatant enzyme. Samples (100 ml) of steady-state cultures (containing approximately 0.1 g [dry weight] of cells) were harvested by centrifugation at 4°C (10 min, 4,000 × *g*). The supernatant was used as a source of extracellular enzyme.

(ii) Cell wall enzyme. The liberation of cell wall-associated enzyme was induced by suspension of the cells in 10 ml of enzyme release buffer (50 mM potassium phosphate, pH 7, 10 mM 2-mercaptoethanol, 10 mM dithiothreitol, 2 mM $MgSO_4$) and incubation for 1 h at 30°C. The suspension was then centrifuged at 4°C and washed twice, first with 5 ml of enzyme release buffer and subsequently with 5 ml of sonication buffer (50 mM potassium phosphate, pH 7, 10 mM $MgSO_4$). Enzyme activities present in the supernatant after incubation of the cells in enzyme release buffer and in the supernatants obtained after washing the cells are designated as preparations of extracellular enzyme formerly trapped in the cell wall. The amount of enzyme released by washing accounted for <1% of the total enzyme produced.

(iii) Cell-bound enzyme. After release of cell wall enzyme and washing, cells were suspended in 5 ml of sonication buffer and sonicated at 4°C with an MSE 150W ultrasonic disintegrator (MSE Ltd., London, England) for 5 min with intermittent periods of cooling. Cell debris was removed by centrifugation at 4°C (15 min, 30,000 × *g*) and suspended in 5 ml of sonication buffer. Enzyme activities present in cell-free extract and resuspended debris were taken as preparations of cell-bound enzyme. The amount of enzyme present in the debris always accounted for <1% of the total enzyme produced.

Occurrence of cell lysis after treatment with enzyme release buffer was routinely checked by following the activity of the constitutive intracellular β-glucosidase, using *ortho*-nitrophenyl-β-D-glycopyranoside (2-NPG) as a substrate. For the assay, enzyme preparation was added to a prewarmed (37°C) solution of 0.1 M potassium phosphate, pH 7, 10 mM KCl, 1 mM $MgCl_2$, and 4 mg of 2-NPG ml^{-1}. The hydrolysis of 2-NPG was followed at 420 nm in an LKB Ultrospec II spectrophotometer (LKB-Produkter, Bromma, Sweden). In no cases could β-glucosidase activity be detected in the preparations of cell wall enzyme. In cell-free extracts, however, irrespective of the growth substrate or dilution rate, high activities of β-glucosidase (0.1 to 1.87 μmol of 2-NPG hydrolyzed min^{-1} mg of cell dry $weight^{-1}$) were present.

Analytical methods. Biomass concentrations were measured by drying to constant weight at 70°C after membrane filtration (0.45 μm; Schleicher & Schüll, Dassel, Federal Republic of Germany) of samples from steady-state cultures.

Glucose and fructose were determined enzymatically, using hexokinase, 6-phosphoglucose dehydrogenase, and phosphoglucose isomerase (glucose/fructose test combination, Boehringer GmbH, Mannheim, Federal Republic of Germany). Sucrose and lactose were determined by the same method after addition of 10 U of either invertase or β-galactosidase (Boehringer) ml^{-1}. Inulin was determined by measuring the amount of fructose and glucose released after acid hydrolysis in sulfuric acid at 100°C as well as after total enzymatic hydrolysis by a preparation of cell wall enzyme from *K. marxianus*. The average chain length of the inulin used was 30 U of fructose plus 1 U of glucose. The molecular weight of the inulin therefore was assumed to be 5,040.

Protein was measured by a modified Bradford method (Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as a standard, according to the instructions of the manufacturer.

Inulinase activity was measured by essentially the same method as described by GrootWassink and Hewitt (9), determining the rate of appearance of fructose and glucose with the Boehringer glucose/fructose test combination in the presence of 2% sucrose or 2% inulin in a 0.1 M sodium acetate buffer, pH 4.5, at 50°C. In all cases enzyme activity was proportional to the amount of enzyme when diluted in 0.1 M sodium acetate, pH 4.5. One unit of inulinase activity is defined as the amount of enzyme catalyzing the liberation of 1 μmol of fructose min^{-1} under the conditions mentioned above. Specific enzyme activities of cultures are given as the sum of the activities from the various fractions and are expressed per milligram of cell dry weight.

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

RESULTS

Choice of organism and cultivation conditions. All strains of *K. marxianus* var. *lactis*, *K. marxianus* var. *marxianus*, and *Kluyveromyces vanudenii* available from the CBS were compared with respect to growth on agar containing either mineral or complex medium and a wide variety of carbon sources. *K. marxianus* var. *marxianus* CBS 6556 was selected for further studies in view of its rapid growth at elevated temperatures on inulin and other sugars. Growth rates of this strain in shake-flask cultures are summarized in Table 1. Except for ethanol, the optimal growth temperature was in the range of 37 to 42°C. Growth in a mineral medium supplemented with pantothenic acid and nicotinic acid was almost as fast as in complex medium. The growth rates of the organism on sugars are the highest reported so far for yeasts.

The response of the organism towards pH was tested in glucose-limited chemostat cultures at a dilution rate of 0.15 h^{-1}. Stable steady states could be obtained between pH 2.6 and 7.5. At the extreme pH values, however, uncoupling of growth and sugar utilization occurred (Fig. 1).

In view of the above results, cultivation was routinely performed at pH 4.5 and 40°C. A mineral medium rather than yeast extract as a source of minerals and vitamins (9, 24) was chosen since this allowed the study of effects of various growth limitations. Moreover, the use of mineral media allows cultivation at high cell densities as required in a commercial process since complex media lead to excessive foaming.

Release of cell wall-associated inulinase. For release of the cell wall enzyme, the methods used by Kidby and Davies (12) and Sommer and Lewis (20) were modified. The effects of various relevant parameters, such as buffer composition, ionic strength of the buffer, buffer pH, and concentration of the sulfhydryl compounds 2-mercaptoethanol and dithiothreitol, were studied. The highest enzyme release was obtained with a 50 mM potassium phosphate buffer, pH 7, containing 10 mM 2-mercaptoethanol and 10 mM dithiothrei-

TABLE 1. Effect of temperature on maximal growth rate of *K. marxianus* CBS 6556[a]

Medium and carbon substrate (2%)	Temp (°C)	Growth rate (h^{-1})
YEPD		
Glucose	33	0.87
Glucose	37	0.89
Glucose	42	0.89
Glucose	45	0.72
Mineral		
Glucose	33	0.69
Glucose	40	0.86
Glucose	42	0.83
Glucose	45	0.53
Inulin	37	0.45
Inulin	42	0.43
Ethanol	33	0.30
Ethanol	40	0.25
Ethanol	45	0.10

[a] Cells were grown in shake flask cultures on the given medium.

tol (enzyme release buffer). The enzyme appeared to be completely stable under these conditions. Prolonged incubation of cells in enzyme release buffer for more than 1 h did not increase the amount of enzyme released. Incubation of cells in a 50 mM potassium phosphate buffer, pH 7, or in a 100 mM sodium acetate buffer, pH 4.5, released 40 to 70% and 30 to 40%, respectively, of the cell wall enzyme within 1 h. These amounts increased during prolonged incubation periods. For this reason, washing of cells before incubation in the enzyme release buffer was omitted. Buffer solutions with other sulfhydryl compounds such as cysteine (13) yielded 60 to 80% of the amount of solubilized enzyme released by the combined activity of 2-mercaptoethanol and dithiothreitol.

The release of cell wall enzyme resulted in an inulinase preparation of considerable purity. It has been reported that a purified inulinase preparation of *K. fragilis* that yielded one band on isoelectric focusing had a specific activity of 2,552 U mg of protein^{-1} (25). Cell wall enzyme obtained from our cultures grown on sucrose at $D = 0.2\ h^{-1}$ (29 U mg of cell dry weight^{-1}) had an activity of 1,739 U mg of protein^{-1}, whereas the enzyme excreted into the culture fluid had a specific activity of 1,310 U mg of protein^{-1}. The activity of inulinase that remained cell bound after incubation in enzyme release buffer could be solubilized by sonication. The specific activity of the enzyme in this fraction was relatively low, 40 U mg of protein^{-1}.

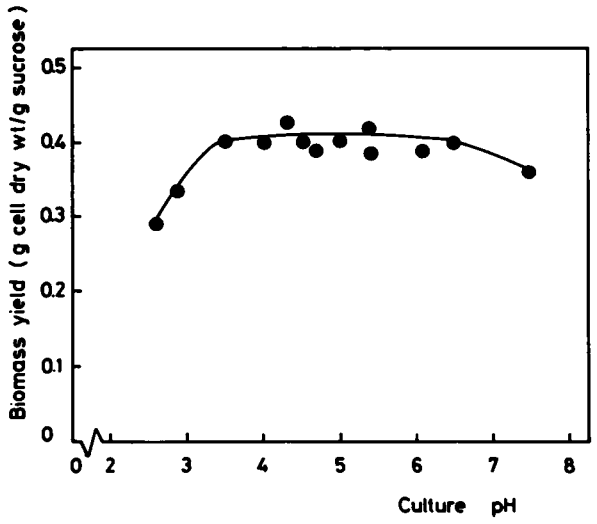

FIG. 1. Effect of culture pH on growth yield of *K. marxianus* CBS 6556 in glucose-limited chemostat cultures. The organism was grown at a dilution rate of 0.15 h^{-1}.

Production and distribution of inulinase of *K. marxianus* in continuous cultures. In carbon- and energy-limited continuous cultures of *K. marxianus* CBS 6556, the highest inulinase yields were obtained with either sucrose or inulin as the limiting substrate (Table 2). Fructose, which is believed to be the primary inducer of inulinase (9), gave an enzyme production half of that observed with sucrose. Growth on glucose or lactose gave very low yields of inulinase. However, considerable amounts of enzyme were produced during growth on the nonfermentable substrate glycerol or ethanol (Table 2).

The levels of inulinase in sucrose-limited chemostat cultures were strongly dependent on the dilution rate. The enzyme levels (sum of cell-bound enzyme, cell wall enzyme, and supernatant enzyme) decreased from a maximum of 52 U mg of cell dry weight^{-1} at $D = 0.1\ h^{-1}$ to 2 U mg of cell dry weight^{-1} at $D = 0.8\ h^{-1}$ (Fig. 2).

The profile of inulinase synthesis in carbon-limited chemostat cultures suggests that the enzyme is regulated by the residual sugar concentration in the culture (2, 3). To substantiate this hypothesis, the effect of nitrogen limitation on cells growing at a fixed rate was studied. Nitrogen-limited growth, obtained with an ammonium sulfate concentration of 200 mg liter^{-1} and a reservoir sucrose concentration of 2.5 g liter^{-1}, resulted in an enzyme activity of 18 U mg of cell dry weight^{-1}. A higher concentration of sucrose in the medium reservoir resulted in a further increase in the residual sugar concentration. This was accompanied by a decrease in inulinase activity to a level comparable to that found during growth under carbon limitation at high dilution rates (Table 3).

So far, no studies have been published on the synthesis of inulinase by yeasts growing in chemostat cultures on mineral media. GrootWassink and co-workers (8, 9, 11, 13) used a complex medium for chemostat cultivation of *K. fragilis*. The medium was composed of yeast extract and a sugar in a 1:2 ratio. Growth of *K. marxianus* CBS 6556 at a low dilution rate on this medium was, however, not carbon limited as indicated by the residual sugar concentration (Table 3). Probably this culture was nitrogen limited because

TABLE 2. Total inulinase activities and distribution in carbon-limited continuous cultures of *K. marxianus* CBS 6556 in mineral medium with 0.25% various carbon substrates

Carbon substrate	Dilution rate (h^{-1})	Total inulinase activity (U mg of cell dry wt^{-1})[a]	% Inulinase in:		
			Supernatant	Cell wall	Cell-bound fraction
Inulin	0.05	58	65	21	14
Inulin	0.15	25	60	26	14
Sucrose	0.10	52	48	32	20
Fructose	0.10	29	51	28	21
Glucose	0.10	3.9	87	8	5
Lactose	0.13	2.8	97	3	0
Glycerol	0.10	9.4	43	37	20
Ethanol	0.10	26	57	31	12

[a] Enzyme activities were measured with sucrose as substrate.

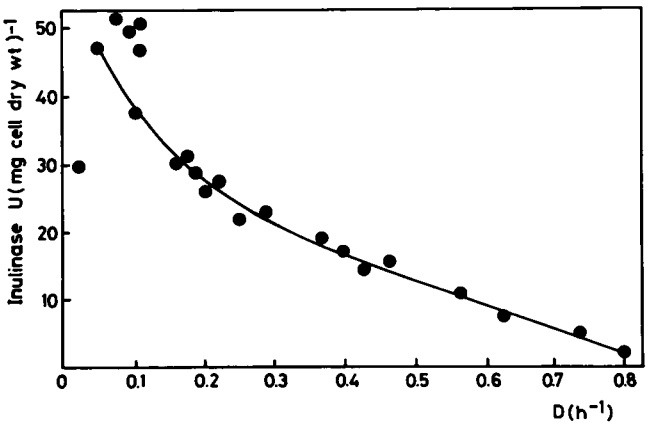

FIG. 2. Effect of dilution rate on total inulinase activity in sucrose-limited chemostat cultures of *K. marxianus* CBS 6556. Enzyme activities were measured with sucrose as substrate.

addition of $(NH_4)_2SO_4$ led to a decrease in the residual sugar concentration (results not shown). There still was, however, incomplete consumption of the sugar, indicating the presence of another limiting nutrient or the presence of a growth-inhibiting factor. The enzyme yields of cultures grown on media with a yeast extract/sucrose ratio of 1:2 or 1:3 were lower than that of a culture grown on a medium composed of yeast extract and sucrose in a ratio of 1:1. Growth on this latter medium led to an inulinase yield (37 U mg of cell dry weight^{-1}) which still was lower than that obtained during carbon-limited growth on the defined mineral medium (52 U mg of cell dry weight^{-1}). Since the residual sugar concentrations in the various cultivation conditions were determined after centrifugation of culture samples, the values listed in Table 3 are an underestimation of the real concentrations, as a consequence of sugar consumption during sample processing. Nevertheless, the data clearly show that the inulinase activity of cultures is negatively correlated with the residual sugar concentration in both mineral and complex media. The results also demonstrate that enzyme production in complex medium is inferior to that in mineral media due to unnoticed limitation even at low yeast extract/sucrose ratios.

TABLE 3. Effect of medium composition on residual sugar concentration and inulinase activity in chemostat cultures of *K. marxianus* CBS 6556

Medium	Dilution rate (h^{-1})	Influent sucrose (g liter^{-1})	Limitation	Residual substrate (mg liter^{-1})[a]	Total inulinase activity (U mg cell dry wt^{-1})[b]
Mineral	0.1	2.5	Carbon	<10	52
Mineral	0.8	2.5	Carbon	110	2.0
Mineral[c]	0.1	2.5	Nitrogen	70	18
Mineral	0.1	5.0	Nitrogen	430	2.8
Mineral	0.1	7.5	Nitrogen	2,620	2.1
Complex[d]	0.1	2.5	Carbon?[e]	30	37
Complex	0.1	5.0	?	90	26
Complex	0.1	7.5	Nitrogen?	690	5.5

[a] Residual substrate was determined after centrifugation of culture samples and is the sum of residual glucose, fructose, and sucrose.
[b] Enzyme activities were measured with sucrose as substrate.
[c] Nitrogen limitation with mineral medium was obtained by lowering the ammonium sulfate concentration to 200 mg liter^{-1}.
[d] The complex medium contained 2.5 g of yeast extract liter^{-1} as a source of minerals and vitamins.
[e] ?, Limitation unknown.

The distribution of enzyme activity among supernatant, cell wall, and cell-bound fractions was dependent on the nature of the growth-limiting carbon substrate. Especially the cell-bound enzyme was present at very low levels when lactose or glucose was used as carbon source (Table 2). Fructose, glycerol, and ethanol gave roughly the same distribution as sucrose and inulin. The distribution of inulinase activity among the fractions was determined at various dilution rates (Fig. 3). Although the amounts of enzyme detected in the supernatant and in the cell wall showed a rather broad variation, the overall picture was that the relative amount of supernatant enzyme remained constant at about 50%. The amount of cell wall enzyme exhibited a slight increase with increasing growth rates up to a dilution rate of 0.6 h^{-1}. The increase in the amount of cell wall enzyme was parallelled by a decrease in the amount of cell-bound enzyme. The cell-bound fraction became nil above a dilution rate of about 0.6 h^{-1}. In contrast to carbon-limited growth on a mineral medium, growth under nitrogen limitation or growth on a complex medium resulted in relatively higher inulinase levels (60 to 80%) in the supernatant and lower levels (6 to 9% of total inulinase activity) in the cell-bound fraction.

The ratio of the activities of the enzyme with sucrose and inulin, determined with 2% substrate solutions at pH 4.5 and 50°C, was 15 ± 3 irrespective of the growth substrate, growth rate, or medium composition.

Effect of temperature and pH on activity and distribution of inulinase. Both production and distribution of the inulinase of *K. marxianus* were affected by the growth temperature. The highest enzyme production was encountered at temperatures between 37 and 42°C (Table 4). Apparently, the temperature range of optimal enzyme production corresponded to the optimal temperature of growth of *K. marxianus* CBS 6556. The enzyme location also varied with the growth temperature. Temperatures below the optimal temperature range of growth and inulinase production gave rise to a larger fraction of enzyme present in the supernatant and a concomitant reduction in the amount of cell wall enzyme (Table 4). The reverse was found at temperatures higher than the optimal growth temperature.

Since a rise in buffer pH stimulated the release of inulinase from the cell wall, it was anticipated that cultivation of the organism at higher pH values would similarly increase the relative amount of enzyme excreted into the culture fluid. When the organism was grown on mineral medium with

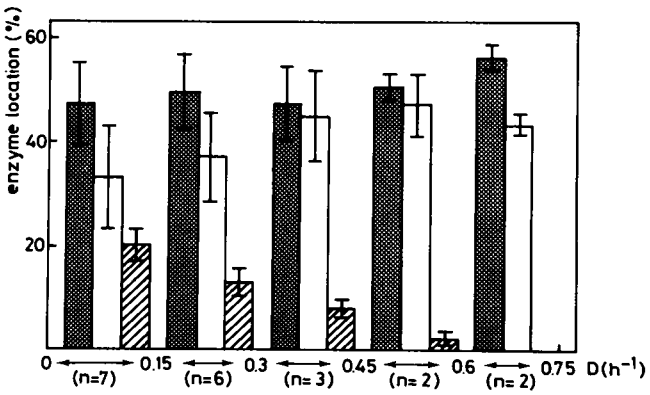

FIG. 3. Effect of dilution rate on distribution of inulinase activity in (▩) supernatant, (□) cell wall, and (▨) cell-bound fractions in sucrose-limited chemostat cultures of *K. marxianus* CBS 6556. Vertical bars represent standard deviations.

TABLE 4. Effect of growth temperature on total inulinase activity and its distribution in sucrose-limited cultures of *K. marxianus* CBS 6556

Temp (°C)	Total inulinase activity (U mg of cell dry wt^{-1})[a]	% Inulinase in:		
		Supernatant	Cell wall	Cell-bound fraction
27	26	62	10	28
35	39	68	14	18
37	45	61	21	18
40	52	48	34	18
42	36	46	33	21
46	12	25	42	33

[a] Enzyme activities were measured with sucrose as substrate.

sucrose at a dilution rate of 0.1 h^{-1} and pH 6.7, the total inulinase activity decreased to approximately half that at pH 4.5. Surprisingly, however, the distribution of the enzyme among the various fractions was the same during growth at pH 6.7 and 4.5.

Effect of temperature and pH on enzyme activity. The activities of inulinase with sucrose and inulin were tested at different temperatures, using a 2% (wt/vol) substrate solution in 0.1 M sodium acetate buffer, pH 4.5. Irrespective of enzyme location or the dilution rate at which the cells were grown, the inulinase showed different temperature optima with sucrose (70°C) and inulin (50°C) (Fig. 4). Thus, the S/I ratio was fairly constant up to about 50°C (almost parallel lines), but greatly increased with higher temperatures. Above 70°C, no activity with inulin was measurable, whereas the activity with sucrose was still high (Fig. 4).

No loss in activity of inulinase was observed when inulinase preparations were incubated for 8 h at temperatures up to 50°C. Incubation at higher temperatures gave rise to heat inactivation of inulinase measured with either sucrose or inulin. At 60°C the half-life was about 30 min.

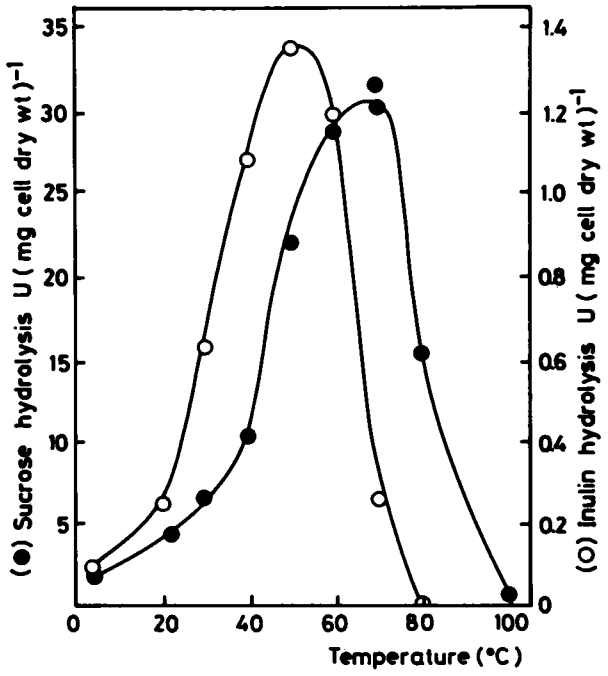

FIG. 4. Effect of temperature on activity of inulinase with sucrose and inulin as substrate. Activities were determined at 50°C, using 2% (wt/vol) solutions of substrate in 0.1 M sodium acetate, pH 4.5.

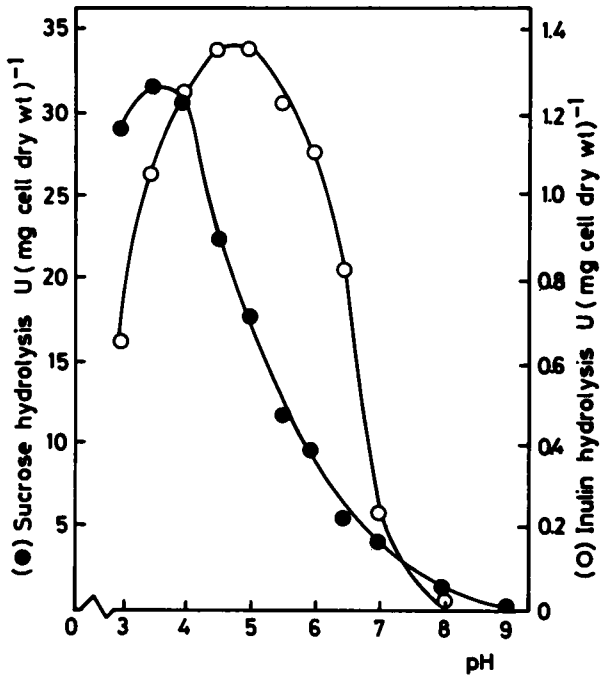

FIG. 5. Effect of pH on activity of inulinase with sucrose and inulin as substrate. Activities were determined at 50°C, using 2% (wt/vol) solutions of substrate in 0.1 M sodium acetate (pH 3 to 5.5) or 0.1 M potassium phosphate (pH 5 to 9).

The effect of pH on the activity of inulinase was tested in standard assay conditions. Irrespective of its origin (cell bound, cell wall, or supernatant), it exhibited a lower pH optimum with sucrose than with inulin as a substrate (Fig. 5). The S/I ratio was dependent on the pH of the assay. The ratio decreased with increasing pH up to 6.5 and again increased at higher pH values. The pH activity profiles were independent of enzyme location or growth rate of the cells.

Kinetic constants of inulinase. Substrates hydrolyzed by yeast β-fructosidases include the oligosaccharides raffinose and stachyose, with chain lengths of three and four sugar moieties, respectively (18). Hydrolysis of sucrose, raffinose, and stachyose followed Michaelis-Menten kinetics. Values for the apparent affinity constant (K_m') and maximal velocity (V') with these oligosaccharides (Table 5) were calculated from plots by the method of Hanes (10). With the chicory inulin preparation (mean chain length, 31 fructose units), however, a separate estimation of K_m' and V' is impossible since a linear relation between the enzyme activity and the substrate concentration was observed up to the saturation

TABLE 5. Kinetic constants of inulinase of *K. marxianus* CBS 6556 for four substrates with different degrees of polymerization[a]

Substrate	K_m' (mM)	V' (U ml^{-1})	V'/K_m'	$(K_{cat}[E_0])/K_m$
Sucrose	15.7	122	7.8	7.7
Raffinose	8.2	38	4.5	4.2
Stachyose	9.7	32	3.2	3.2
Inulin				1.5

[a] Inulinase was isolated from cells grown in a sucrose-limited continuous culture at D = 0.2 h^{-1}.

concentration of this substrate. Assuming that hydrolysis of inulin also exhibits Michaelis-Menten kinetics, it follows that the maximal chicory inulin concentration that can be achieved is well below the apparent affinity constant. Under these conditions, the Michaelis-Menten equation can be represented by: $V = K_{cat}[E] \cdot S/K_m$, where $[E]$ represents the concentration of free enzyme. At substrate concentrations well below K_m, the concentration of free enzyme approximates $[E_0]$, the total enzyme concentration. Therefore, the behavior of the enzyme towards the different substrates can be compared by using the slopes of the plots of V versus $[S]$ at equal total enzyme concentrations. The slopes represent $K_{cat}[E_0]/K_m$, which, under these conditions, should be equal to the calculated V'/K_m'. With this method, it appears that sucrose is a better substrate than inulin for inulinase and that the enzyme specificity (K_{cat}/K_m) decreases with increasing chain length (Table 5). The apparent affinity constant K_m, however, was lower for the oligosaccharides raffinose and stachyose than for sucrose. The kinetics of the enzyme were independent of its origin. No difference in V'/K_m' values was observed when supernatant, cell wall, and cell-bound enzyme were compared.

DISCUSSION

Regulation of enzyme synthesis. In previous studies on the formation of inulinase by *Kluyveromyces* spp. in batch (17, 18) and continuous (8, 9) cultures, it was concluded that the enzyme is regulated by induction and repression. However, in various cases high enzyme levels were encountered in the absence of inducer (Table 6). Results from batch cultures (Table 6) should be interpreted cautiously, since in these cultures enzyme levels depend on the time of harvesting and thus are subject to large variations.

Use of chemostat cultivation permits a more precise analysis of the regulation of inulinase synthesis, provided that defined media are used and the limiting nutrient is known. Inulinase production by *K. marxianus* CBS 6556 decreased with increasing dilution rate (Fig. 2). The same relationship between dilution rate and inulinase production was reported for *K. fragilis* ATCC 12424, a yeast now classified as *K. marxianus* var *marxianus*. This was, however, determined in complex medium over a very small range of dilution rates (18). The decrease in inulinase activity with increasing dilution rates is primarily caused by the increase in residual sugar concentration at higher dilution rates. This was also evident from an analysis of enzyme production in cultures grown under different degrees of nitrogen limitation at a fixed dilution rate. The presence of higher concentrations of residual substrate led to lower enzyme activities (Table 2). These observations indicate that the enzyme is regulated by catabolite repression (2, 3). The finding that ethanol and, to a lesser extent, glycerol also gave rise to fairly high levels of inulinase (Table 1) is in contradiction to the suggested inducible nature of the enzyme (9). Rather, derepression of enzyme synthesis is likely to occur when cells are grown on these substrates.

TABLE 6. Published data on inulinase production by yeasts during growth on various carbon sources

Carbon source	Enzyme level (%)[a] in given organism and cultivation conditions				
	S. fragilis 351, batch[b]	*K. fragilis* ATCC 12424[c]		*K. marxianus* UCD 55-82, batch[d]	*K. marxianus* CBS 6556, chemostat[e]
		Batch	Chemostat		
Inulin	1,000	500	ND	294	111
Sucrose	100	100	100	100	100
Fructose	340	100	111	88	54
Glucose	170	100	33	79	8
Lactose	ND	50	<1	ND	6
Galactose	170	50	1	ND	ND
Ethanol	ND	100	<1	ND	50
Glycerol	ND	150	ND	ND	18

[a] For comparison, enzyme levels are presented as percentages of the activities of cells grown on sucrose as carbon source. ND, Not determined.
[b] Snyder and Phaff (18); complex medium with 2% substrate.
[c] GrootWassink and Hewitt (9); complex medium with 1% substrate.
[d] Parekh and Margaritis (17); complex medium with 1% substrate.
[e] This study; carbon-limited cultures on mineral medium with 0.25% substrate.

Regulation of another yeast β-fructosidase, invertase, has been more extensively studied. In *Saccharomyces cerevisiae*, synthesis of this enzyme is only subject to carbon catabolite repression and does not require a specific inducer (7, 14, 15). This conclusion was based on invertase levels in cultures of wild-type *S. cerevisiae*, enzyme levels in cultures of regulatory mutants, and mRNA contents of cells under repressive and derepressive growth conditions (4, 15).

K. marxianus CBS 6556 has a maximum inulinase yield of about 50 U mg of cell dry weight^{-1} when grown in sucrose-limited continuous cultures at low dilution rates (Table 2; Fig. 2). This inulinase yield is among the highest reported so far. Most research on inulinase formation and regulation, reviewed by Vandamme and Derycke (24), has been performed by using cultivation on complex media. In our experience a complex medium is less favorable to enzyme formation than a defined mineral medium (Table 3). Probably this was caused by the absence of true carbon limitation when yeast extract was used as a source of nitrogen and vitamins.

In our studies with mineral medium, only the effects of carbon- and nitrogen-limited growth on inulinase production were determined. It is possible, however, that other limitations may have effects on inulinase production. In this respect, the work of Toda and co-workers is of importance. They reported a pronounced positive effect of phosphate limitation on the production of invertase by the yeast *Saccharomyces carlsbergensis*, which overruled the repressive effect of high residual sugar concentration (22, 23).

Distribution of inulinase. The distribution of inulinase over supernatant, cell wall, and cell-bound fractions was dependent on the (i) nature of the carbon-limiting substrate, (ii) dilution rate, (iii) medium composition (mineral or complex), and (iv) growth temperature. As already observed by Lam and GrootWassink (13) for *K. fragilis*, irrespective of the dilution rate, about half of the total amount of enzyme produced in sucrose-limited continuous cultures of *K. marxianus* CBS 6556 was present in the culture supernatant. The distribution of the other half of the enzyme between cell wall and cell-bound fractions, however, differed with the dilution rate. The cell-bound fraction decreased with increasing dilution rates (Fig. 3). Carbon-limited growth on glucose or lactose or nitrogen-limited growth (in both mineral and complex media) with sucrose resulted in high percentages of inulinase present in the culture supernatant and low amounts of cell wall and cell-bound enzyme (Table 2). Retention of inulinase in the cell wall thus seems to be less pronounced when cells are grown under conditions that result in a low level of enzyme. It remains to be elucidated whether this is a consequence of differences in cell wall composition or differences in enzyme structure.

As yet it is not clear whether the cell-bound enzyme

represents a tightly bound enzyme located outside the cytoplasmic membrane or, comparable to the invertase of *Saccharomyces* spp. (14), an intracellular enzyme. In this respect it is relevant to mention the findings of Esmon et al. (5) and Tammi et al. (21) on the multimeric structure and excretion of invertase in Saccharomyces spp. These authors demonstrated that invertase is an octameric complex of four invertase dimers throughout the secretory process and that this complex appears to play a role in the retention of invertase within the cell wall. Both invertase released into the culture fluid and the fraction of cell wall invertase that can be released after treatment of the cells with sulfhydryl compounds are composed of invertase dimers with the same kinetic properties as the octamer. Conversion of octamer to dimer is promoted by several treatments (e.g., sonication) but not by treatment with 2-mercaptoethanol. Even when intact cells are treated with 2-mercaptoethanol, the octamer is preferentially retained in the cell wall and release requires cell wall disruption (21). It is thus possible that the distribution of inulinase in *K. marxianus*, like that of invertase in *Saccharomyces* spp., may depend on the subunit composition of the enzyme.

Kinetic properties of inulinase. In standard assay conditions, the S/I ratio of the β-fructosidase of *K. marxianus* CBS 6556 was 15, which would make it a true inulinase (24). Temperature and pH optima (Fig. 4 and 5) differed with sucrose or inulin as substrate. This phenomenon has also been observed for β-fructosidases from other *Kluyveromyces* strains (18, 25) and from other yeasts (1). As a consequence, S/I ratios cannot be compared without prior knowledge of the dependence of enzyme activity on pH and temperature. Moreover, the value of the S/I ratio is influenced by the substrate concentration and by the origin of the inulin preparation used.

Contrary to bacteria and molds, all yeast β-fructosidases show a restricted mode of action. They all remove fructose moieties exowise, and all have the capability of hydrolyzing both sucrose and inulin (6). The question remains as to whether differences in values such as S/I ratios and apparent kinetic constants for substrates with undefined molecular weights provide sufficient evidence for the classification of yeast β-fructosidases into two different enzymes: inulinase and invertase.

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LITERATURE CITED

1. **Beluche, I., J. P. Guiraud, and P. Galzy.** 1980. Inulinase activity of *Debaromyces canterellii*. Folia Microbiol. **25**:32–39.
2. **Bull, A. T.** 1972. Environmental factors influencing the synthesis and excretion of exocellular macromolecules. J. Appl. Chem. Biotechnol. **22**:261–292.
3. **Dean, A. C. R.** 1972. Influence of environment on the control of enzyme synthesis. J. Appl. Chem. Biotechnol. **22**:245–259.
4. **Elorza, M. V., J. R. Villanueva, and R. Sentandreu.** 1977. The mechanism of catabolite inhibition of invertase by glucose in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **475**:103–112.
5. **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.
6. **Fuchs, A., J. M. de Bruijn, and C. J. Niedeveld.** 1985. Bacteria and yeasts as possible candidates for the production of inulinases and levanases. Antonie van Leeuwenhoek J. Microbiol. Serol. **51**:333–351.
7. **Gascon, S., and P. Ottolenghi.** 1972. Influence of glucose concentration of the medium on the invertase content of a strain of *Saccharomyces* bearing the SUC 2 gene. C. R. Trav. Lab. Carlsberg **39**:15–24.
8. **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.
9. **GrootWassink, J. W. D., and G. M. Hewitt.** 1983. Inducible and constitutive formation of β-fructofuranosidase (inulase) in batch and continuous cultures of the yeast *Kluyveromyces fragilis*. J. Gen. Microbiol. **129**:31–41.
10. **Hanes, C. S.** 1932. Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. Biochem. J. **26**:1406–1421.
11. **Hewitt, G. M., and J. W. D. GrootWassink.** 1984. Simultaneous production of inulase and lactase in batch and continuous cultures of *Kluyveromyces fragilis*. Enzyme Microb. Technol. **6**:263–270.
12. **Kidby, D. K., and R. Davies.** 1970. Thiol induced release of invertase from cell walls of *Saccharomyces fragilis*. Biochim. Biophys. Acta **201**:261–266.
13. **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.
14. **Lampen, J. O.** 1971. Yeast and Neurospora invertases, p. 291–302. *In* P. D. Boyer (ed.), The enzymes, vol. 5, 3rd ed. Academic Press, Inc., New York.
15. **Mormeneo, S., and R. Sentandreu.** 1986. Molecular events associated with glucose repression of invertase in *Saccharomyces cerevisiae*. Antonie van Leeuwenhoek J. Microbiol. Serol. **52**:15–24.
16. **Negoro, H., and K. Kito.** 1973. β-Fructofuranosidase from *Candida kefyr*. J. Ferment. Technol. **51**:96–102.
17. **Parekh, S., and A. Margaritis.** 1985. Inulinase (β-fructofuranosidase) production by *Kluyveromyces marxianus* in batch culture. Appl. Microbiol. Biotechnol. **22**:446–448.
18. **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.
19. **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.
20. **Sommer, A., and M. J. Lewis.** 1971. Effect of dithiothreitol on yeast, sphaeroplast formation and invertase release. J. Gen. Microbiol. **68**:327–335.
21. **Tammi, M., L. Ballou, A. Taylor, and C. E. Ballou.** 1987. Effect of glycosylation on yeast invertase oligomer stability. J. Biol. Chem. **262**:4395–4401.
22. **Toda, K., I. Yabe, and T. Yamagata.** 1982. Kinetics of yeast growth and enzyme syntheses in a phosphate-limited continuous culture. Eur. J. Appl. Microbiol. Biotechnol. **16**:10–16.
23. **Toda, K., I. Yabe, and T. Yamagata.** 1982. Invertase and phosphatase of yeast in a phosphate-limited continuous culture. Eur. J. Appl. Microbiol. Biotechnol. **16**:17–22.
24. **Vandamme, E. J., and D. G. Derycke.** 1983. Microbial inulinases: fermentation process, properties, and applications. Adv. Appl. Microbiol. **29**:139–176.
25. **Workman, W. E., and D. F. Day.** 1983. Purification and properties of the β-fructofuranosidase from *Kluyveromyces fragilis*. FEBS Lett. **160**:16–20.
26. **Workman, W. E., and D. F. Day.** 1984. The cell wall-associated inulinase of *Kluyveromyces fragilis*. Antonie van Leeuwenhoek J. Microbiol. Serol. **50**:349–353.