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Production and Localization of β-Fructosidase in Asynchronous and Synchronous Chemostat Cultures of Yeasts

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In synchronized continuous cultures of *Saccharomyces cerevisiae* CBS 8066, the production of the extracellular invertase (EC 3.2.1.26) showed a cyclic behavior that coincided with the budding cycle. The invertase activity increased during bud development and ceased at bud maturation and cell scission. The cyclic changes in invertase production resulted in cyclic changes in amounts of invertase localized in the cell wall. However, the amount of enzyme invertase present in the culture liquid remained constant throughout the budding cycle. Also, in asynchronous continuous cultures of *S. cerevisiae*, the production and localization of invertase showed significant fluctuation. The overall invertase production in an asynchronous culture was two to three times higher than in synchronous cultures. This could be due to more-severe invertase-repressive conditions in a synchronous chemostat culture. Both the intracellular glucose-6-phosphate concentration and residual glucose concentration were significantly higher in synchronous chemostat cultures than in asynchronous chemostat cultures. In the asynchronous and synchronous continuous cultures of *S. cerevisiae*, about 40% of the invertase was released into the culture liquid; it has generally been believed that *S. cerevisiae* releases only about 5% of its invertase. In contrast to invertase production and localization in the chemostat cultures of *S. cerevisiae*, no significant changes in inulinase (EC 3.2.1.7) production and localization were observed in chemostat cultures of *Kluyveromyces marxianus* CBS 6556. In cultures of *K. marxianus* about 50% of the inulinase was present in the culture liquid.

In yeasts, enzymes that hydrolyze oligosaccharides are located in the cell wall. Depending on culture conditions and yeast species, variable amounts of enzyme may be released in the culture medium (9, 14). Two mechanisms for the retention of these enzymes in the cell wall have been proposed. One is based on the assumption that these glycoproteins are associated with phosphomannan components of the outer cell wall region by either covalent or noncovalent linkages (9). The alternative proposal is that the outer layers of the cell wall function as a permeability barrier (7). The barrier hypothesis was affirmed by the finding that the localization of the two β-fructosidases known in yeasts, namely, invertase (EC 3.2.1.26) produced by *Saccharomyces cerevisiae* and inulinase (EC 3.2.1.7) produced by *Kluyveromyces marxianus* (14, 16), was dependent on the degree of oligomerization of the enzymes. Both enzymes are excreted into the culture liquid as a dimer. The enzyme retained in the cell wall is a tetramer in the case of inulinase and an octamer in the case of invertase (4, 13).

The secretion of glucanase, acid phosphatase, and invertase follows the pattern of cell surface growth in *S. cerevisiae* (2, 5, 17). During budding an increased amount of transport vesicles containing both newly synthesized cell wall material and extracellular enzymes is localized at the developing bud. As a result of exocytosis, newly secreted enzymes mainly reside in the cell wall surrounding the developing bud. The secretion continues until growth of the daughter cell is complete, suggesting a close relationship between the cell division cycle and enzyme secretion (5). The developing wall of the bud is less rigid than that of the mother cell (15). Therefore, the developing wall could temporarily contain larger pores and be more permeable to the glycoproteins. The release of the glycoproteins into the culture liquid could thus be highest at the stage of bud development. However, the possibility that release of extracellular enzymes into the culture medium is related to the budding cycle has not been tested.

A convenient method for the study of a relation between enzyme production and budding cycle of yeasts is the use of oscillating continuous cultures. In these cultures part of the cell population exhibits synchronized budding. This method has proved useful for the study of the synthesis of intracellular enzymes in relation to the budding cycle (1, 6, 8, 20). In this paper, the production and release of extracellular enzymes in synchronous and asynchronous continuous cultures of yeasts are investigated by using two well-known β-fructosidases, namely, invertase and inulinase, as model enzymes.

MATERIALS AND METHODS

Yeast strains and growth conditions. *Kluyveromyces marxianus* var. *marxianus* CBS 6556 and *S. cerevisiae* CBS 8066 were obtained from the Yeast Division of the Centraal Bureau voor Schimmelcultures (CBS), Delft, The Netherlands. Yeasts were maintained on YEPD agar slopes (yeast extract, 10 g; Bacto-Peptone, 10 g; and glucose, 20 g [each per liter of demineralized water]). The organisms were grown in a laboratory fermenter with a working volume of 1 liter (Applikon, Schiedam, The Netherlands). The pH was maintained at the desired value by automatic addition of 1 M KOH and 0.5 M H_2SO_4. *S. cerevisiae* was cultivated at D = 0.18 h^{-1}, pH 5.0, and a temperature of 30°C; *K. marxianus*

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was cultivated at $D = 0.1\ h^{-1}$ or $D = 0.18\ h^{-1}$, pH 4.5, and a temperature of 40°C. For carbon- and energy-limited growth, the mineral medium of Van Urk et al. (18) was used. For carbon- and energy-limited growth, sucrose was added to the mineral salts medium to a final concentration of 5 g $liter^{-1}$.

Oscillating continuous cultures. *S. cerevisiae* CBS 8066 was grown batchwise in the fermenter on the mineral salts medium containing glucose (5 g/liter). After entering the stationary phase, the continuous feed of medium with sucrose was started. In three of eight trials, the transition from batch to continuous mode led to stable oscillations in the carbon dioxide production rate. The oscillations persisted for a minimum period of 2 days and a maximum period of 8 days. Since a substantial part of the cultures appeared to have become synchronized in budding, such cultures were labeled synchronous.

Sampling. Oscillating, synchronous chemostat cultures and nonoscillating, asynchronous continuous cultures of yeasts were sampled only after five volume changes following start-up of continuous culture conditions. In order to maintain steady-state conditions, preserved samples (30 ml) were taken from the outlet (sampling time, 10 min). The outlet was received in a cooled flask (4°C).

Yeast cells in a culture sample were counted under a light microscope (×1,000) by using a Burkner hemocytometer, and the percentage of budding cells in the population was calculated. From the moment the bud became visible to the scission of the daughter cell, the cells were considered to be budding (9).

Gas analysis. Analysis of CO_2 in fermenter outlet gas was performed on line with a Beckman 864 infrared carbon dioxide analyzer (Beckman Instruments, Fullerton, Calif.) and registered with a Kipp BD 41 recorder (Kipp & Zn., Delft, The Netherlands). For calculation of carbon dioxide production (Q_{CO_2}), the method described by Van Urk et al. (19) was used.

Fractionation of cultures for inulinase and invertase assays. Samples (10 ml) were fractionated into three enzyme preparations by the method of Rouwenhorst et al. (14). Enzyme present in the culture liquid is designated supernatant enzyme. Induced release of extracellular enzyme from the cell wall was obtained by suspending 7 mg of cells in 1 ml of a potassium phosphate buffer (50 mM, pH 7) containing 10 mM 2-mercaptoethanol and 10 mM dithiothreitol and incubating the suspension for 1 h at 30°C. The suspension was then centrifuged at 4°C. Enzyme activity present in the supernatant after incubation of the cells in buffer with sulfhydryl compounds is designated cell wall enzyme.

After the induced release of cell wall enzyme and washing, cells were resuspended in 1 ml of phosphate buffer (50 mM, pH 7) and sonicated at 4°C 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 1 ml of phosphate buffer. Cytoplasmic enzyme activity and extracellular enzyme activity that could be solubilized from the cell walls only by means of sonication are designated cell-bound enzyme and were measured in the cell extracts. Thus, no distinction is made between strongly cell wall-retained enzyme and intracellular enzyme. Enzyme activities present in resuspended cell debris always accounted for less then 1% of the total enzyme produced.

Analytical methods. Biomass concentrations were established by total organic carbon analysis. A Beckman 915B Tocamaster (Beckman) was used to determine the carbon content in culture suspensions and culture supernatant; the carbon content of yeast was obtained from the difference. Yeast dry weight was calculated by using an assumed carbon content of 50%. As a standard, a 2.137-g $liter^{-1}$ solution of anhydrous potassium biphthalate was used.

Residual sugar was determined after centrifugation of culture samples. Samples were collected at 4°C for 1 min from the fermenter outlet. This sampling method was required in order to preserve steady-state conditions. As a result, the values for residual sugar concentration will be an underestimation of the real value because of sugar consumption during sample processing. Hexose-6-phosphate concentrations were determined in the cell extracts and related to the cell dry weight, assuming a 100% efficiency of cell disruption during sonication.

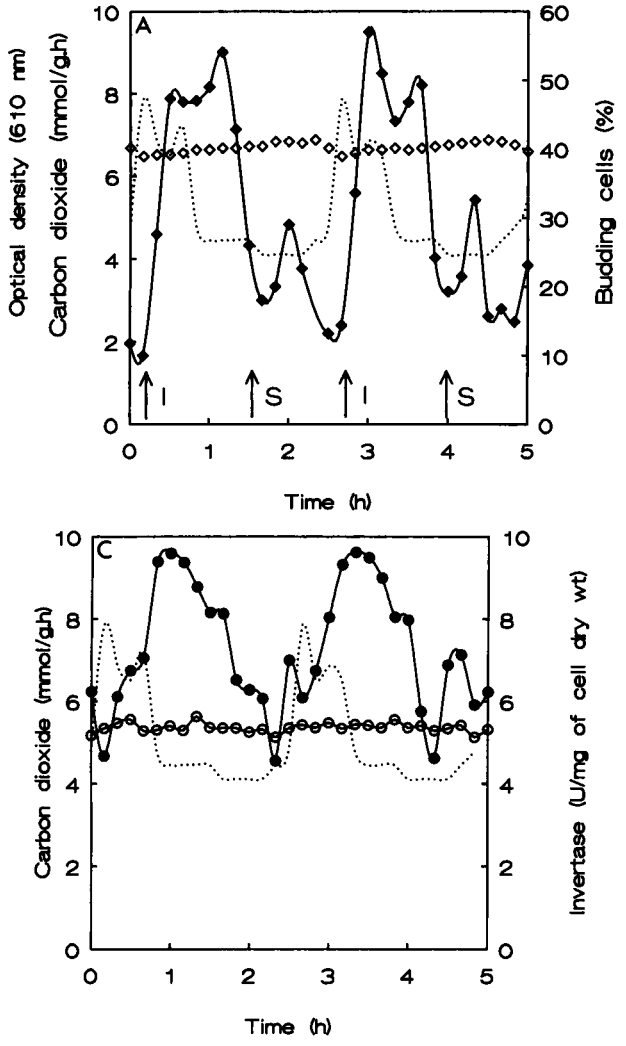
Glucose and fructose were determined enzymatically by using hexokinase, 6-phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase (glucose-fructose test combination; Boehringer GmbH, Mannheim, Federal Republic of Germany). Sucrose was determined by the same method after addition of 10 U of invertase (Boehringer) ml^{-1}. Hexose-6-phosphate was determined enzymatically by using glucose-6-phosphate dehydrogenase. Because of the presence of phosphoglucose isomerase in cell extracts, no distinction could be made between glucose-6-phosphate and fructose-6-phosphate.

Sucrose-hydrolyzing activities of invertase and inulinase were measured by the method described previously (14). One unit of invertase or inulinase activity is defined as the amount of enzyme catalyzing the liberation of 1 μmol of fructose min^{-1} at pH 4.5 and 50°C. Specific activities were related to cell dry weight. As the sampling time, the mean time of collection was used.

Chemicals. Yeast extract and Bacto-Peptone were from Difco Laboratories, Detroit, Mich. Sucrose and 2-mercaptoethanol were from Baker Chemicals, Deventer, The Netherlands. Dithiothreitol was purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Oscillating cultures of *S. cerevisiae*. Oscillations in oxygen consumption and carbon dioxide production of *S. cerevisiae* cultures reflect the occurrence of synchronized budding in the culture. These oscillations can occur spontaneously after transition of a culture from batch to continuous cultivation (11) or can be induced by stepwise changes in either dilution rate or glucose feed rate (1, 12, 20). Spontaneously oscillating continuous cultures of *S. cerevisiae* CBS 8066 growing on sucrose were obtained after transition from stationary batch cultures to continuous mode. At a dilution rate of 0.18 h^{-1}, stable oscillations in carbon dioxide production rate with a period of 2.5 h were observed (Fig. 1). During the oscillations the percentage of budding cells, the optical density at 610 nm, and the intracellular hexose-6-phosphate concentration were determined. The behavior of an oscillating *S. cerevisiae* culture is well documented (1, 6, 8, 20). The oscillations in oxygen consumption and carbon dioxide production in fully aerobic cultures of *S. cerevisiae* are a result of a cyclic budding of part of the yeast population. The development of a yeast cell can be subdivided into a single-cell phase and a double-cell phase (Fig. 1A). During the single-cell phase (Fig. 1A, S), the cells metabolize glucose oxidatively and accumulate reserve carbohydrates such as glycogen and trehalose. At the beginning of budding (Fig. 1A, I), a rapid degradation of reserve carbohydrates occurs. During this period, the yeast changes to a fermentative



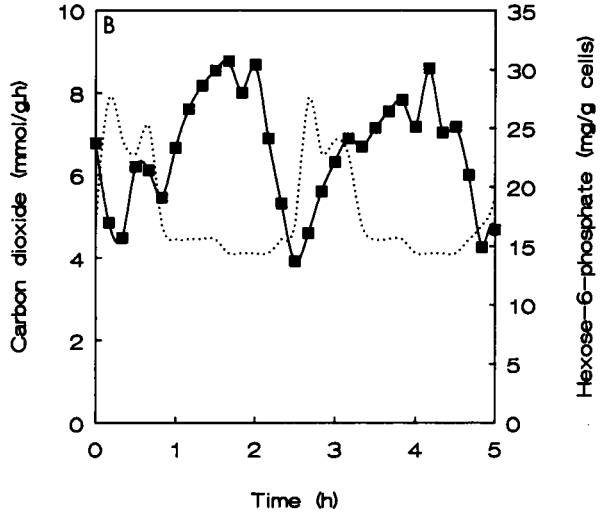


FIG. 1. Synchronous growth of *S. cerevisiae* under sucrose limitation in an oscillating continuous culture at a dilution rate of 0.18 h^{-1} (mean generation time, 3.85 h) at pH 5 and 30°C. (A) Course of culture biomass (◇) and percentage of double cells (◆). (B) Course of glucose-6-phosphate in cell extracts of the culture (■). (C) Production of invertase during the oscillating culture divided in supernatant invertase (○) and cell-bound invertase (●) (see text). The pattern of the carbon dioxide production rate is presented in each panel by a dashed line.

metabolism and ethanol is secreted into the medium (1, 6, 20). The production of ethanol coincides with a marked increase in carbon dioxide production and a slight decrease in biomass in the culture (Fig. 1A). This sudden change in glucose metabolism is then followed by an oxidative breakdown of the previously formed ethanol. The highest percentage of double cells is attained when the carbon dioxide production rate drops to nearly its original value. Of the total cell population a maximum of 56% of budding cells was observed (Fig. 1A). During the oscillations, two subpopulations of *S. cerevisiae* showed a synchronized cell cycle (Fig. 1A). The first subpopulation had peaks in budding cells of, on average, 50% after the carbon dioxide production had dropped to its minimal values, i.e., at 0.8 to 1 h and 3.2 to 3.5 h; the second subpopulation had peaks in budding cells of, on average, 30% at 1.9 and 4.3 h. Although there are two synchronously growing subpopulations, only the first correlates with changes in the production and localization of invertase.

The hexose-6-phosphate concentration showed an oscillating behavior with minimal and maximal concentrations of 14 and 32 mg g of cells (dry weight)$^{-1}$, respectively (Fig. 1B). The residual sugar concentration in the oscillating culture was 26.6 ± 2.8 mg liter^{-1}. A summary of the parameters determined in synchronous and asynchronous continuous cultures is given in Table 1.

Localization of invertase in synchronous chemostat cultures of *S. cerevisiae*. During the synchronized continuous culture the distribution of invertase over supernatant, cell wall, and cell-bound fractions was determined. Treatment of *S. cerevisiae* cells with sulfhydryl compounds did not result in a marked release of invertase that had been retained within the cell wall. Only about 0.3 U mg of cells (dry weight)$^{-1}$, i.e., 4% of total invertase produced, was present in this fraction (Table 1). The insensitivity of *S. cerevisiae* to treatment with sulfhydryls has long been known (3, 4), and release of cell wall-retained invertase can be achieved only by complete removal of the cell wall during spheroplast formation (9). Spheroplasts still contain sucrose-hydrolyzing activity due to an intracellular invertase which amounts to about 5% of total invertase activity (5). However, in the figures presented here, no distinction is made between cell wall-retained invertase and intracellular invertase. The cell extracts (cell-bound invertase) contained both these invertases. The invertase in this cell-bound fraction showed a marked oscillating behavior in synchronously dividing cultures (Fig. 1C). The cyclic pattern of cell-bound invertase parallelled the cyclic pattern of the percentage of budding cells in the culture. The cell-bound invertase increased with increasing percentages of budding cells, up to a maximum of 9.6 U mg of cells (dry weight)$^{-1}$, and decreased to 4.6 U mg of cells (dry weight)$^{-1}$, when bud maturation and cell division took place (Fig. 1A and C). The invertase activity correlated negatively to the hexose-6-phosphate concentration.

Irrespective of the amount of invertase present in the cells, the invertase released into the culture liquid remained constant at 5.3 ± 0.3 U mg of cells (dry weight)$^{-1}$ throughout the budding cycle. During continuous cultivation, a constant amount of cells and culture liquid is removed from the culture. In the case of cessation of invertase synthesis during

TABLE 1. Various parameters in synchronous and asynchronous continuous cultures[a]

Culture	Q_{CO_2} (mmol/g · h^{-1})	Glucose-6-phosphate (mg/g [dry wt])	Residual sugar[b] (mg/liter)	β-Fructosidase activity[c] (U/mg [dry wt])		
				Supernatant	Cell wall	Cell bound
S. cerevisiae CBS 8066						
Synchronous	4.0–7.9	14–32	26.6 ± 2.8	5.3 ± 0.2	0.3 ± 0.07	4.6–9.6
Asynchronous	4.1 ± 0.4	4.9 ± 1.1	7.9 ± 0.6	19.3 ± 0.7	0.4 ± 0.04	20.0–28.8
K. marxianus CBS 6556, asynchronous	4.0 ± 0.3	ND[d]	ND	29.6 ± 1.7	17.6 ± 2.2	8.4 ± 0.9

[a] *S. cerevisiae* was grown at $D = 0.18\ h^{-1}$. *K. marxianus* was grown at $D = 0.1\ h^{-1}$.
[b] Residual sugar is an underestimation of the real value. By using fast sampling, a residual concentration of 19.8 mg/liter for *S. cerevisiae* CBS 8066 was established (18).
[c] Activities measured were invertase in *S. cerevisiae* and inulinase in *K. marxianus*. Cell wall activities measured enzyme released by sulfhydryls. Cell-bound activities measured cell wall enzyme plus intracellular enzyme in cell extracts.
[d] ND, Not determined.

bud maturation and cell scission, both the cell-bound invertase and supernatant invertase are expected to decrease, according to washout kinetics. However, as already mentioned above, the amount of supernatant invertase remained constant. This implies that there should be an increased release of invertase from the cells. The observed decrease in cell-bound invertase activity (Fig. 1C) could then be explained by the release of invertase into the culture liquid. However, if invertase synthesis ceases, the total amount of invertase present in the culture still should follow washout kinetics. The decline in total invertase activity during bud maturation and the theoretical washout are presented in Fig. 2. The decrease in total invertase activity did not completely follow theoretical washout kinetics (Fig. 2). Only during the first 40 to 50 min did the decrease in total invertase appear to follow washout kinetics. From 50 min onwards, invertase started to decline more rapidly than can be expected from washout kinetics. Apparently, during cell scission either a gradual inactivation or denaturation of invertase took place.

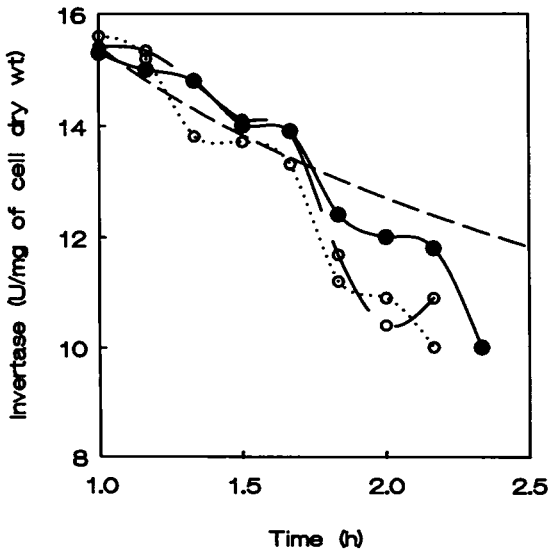


FIG. 2. Decline in total invertase activity during the disappearance of double cells; three separate oscillations in a synchronous, sucrose-limited, continuous culture of *S. cerevisiae* CBS 8066 at a dilution rate of 0.18 h^{-1} were monitored. The dashed line represents the theoretical washout of invertase.

Localization of invertase in asynchronous chemostat cultures of *S. cerevisiae*. After several days, the oscillations in carbon dioxide production disappeared and apparently the culture became asynchronous in cell division cycle. For comparison with the behavior of a synchronous culture, the production and localization of invertase in the asynchronous continuous culture were followed after five extra volume changes of the fermenter. As evidenced by the constancy in carbon dioxide production (4.1 ± 0.4 mmol/g · h^{-1}), optical density of the culture at 610 nm (7.12 ± 0.07), and residual substrate concentration (7.9 ± 0.6 mg $liter^{-1}$), the culture then represented a steady-state continuous culture. During the steady state, the activities of supernatant invertase and cell wall invertase remained unchanged at 19.3 ± 0.7 and 0.42 ± 0.04 U mg of cells (dry weight)$^{-1}$, respectively (Table 1; Fig. 3). The cell-bound invertase activity showed a remarkable variation, with activities between 20 and 28.8 U mg of cells (dry weight)$^{-1}$. This variation in cell-bound invertase activity was not due to the fractionation or assay methods. The coefficient of variation in 10 independent determinations of a single measurement starting at the fractionation of a culture suspension was 5.2%.

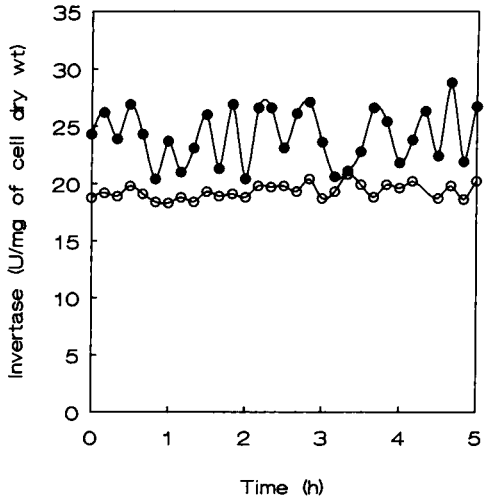


FIG. 3. Course of supernatant invertase (○) and the sum of cell wall and cell-bound invertase (●) in an asynchronous, sucrose-limited, continuous culture of *S. cerevisiae* CBS 8066 at pH 5, 35°C, and a dilution rate of 0.18 h^{-1}.

The total invertase activity was between 38.8 and 48.6 U mg of cells (dry weight)$^{-1}$. This is about three times the amount of invertase found in synchronous *S. cerevisiae* culture. The variation in total invertase was only due to the variation in cell-bound invertase because the amount of supernatant invertase remained unchanged.

Localization of inulinase in asynchronous chemostat cultures of *K. marxianus*. The extracellular inulinase of *K. marxianus* is strongly related to the invertase of *S. cerevisiae*. In order to determine whether the budding cycle-related variation in extracellular β-fructosidase production and localization also occurs in *K. marxianus*, we tried to obtain synchronous cultures of this yeast and determine inulinase activities. Various attempts to obtain an oscillating culture of *K. marxianus* CBS 6556 were unsuccessful. Only after stepwise increases in dilution rate up to 0.89 h^{-1}, at which the mean generation time almost equals the minimal time necessary for budding (11, 20), were oscillations in carbon dioxide production of a *K. marxianus* culture observed. However, because of inulinase repression at this high dilution rate (14), the amount of inulinase was too low, viz., 0.5 to 2 U mg of cells (dry weight)$^{-1}$, to obtain reproducible results with respect to inulinase distribution. Analogous to the invertase in asynchronous steady-state cultures of *S. cerevisiae* CBS 8066, the production and localization of inulinase was monitored during asynchronous steady-state cultures of *K. marxianus* growing on sucrose at $D = 0.1$ h^{-1} (Table 1). In contrast to *S. cerevisiae*, treatment of *K. marxianus* cells with sulfhydryl compound led to a release of cell wall inulinase. The inulinase activities in cell wall fraction and cell-bound fraction were 17.6 ± 2.2 and 8.4 ± 0.9 U mg of cells (dry weight)$^{-1}$, respectively. In contrast to a steady-state culture of *S. cerevisiae*, there was no significant change in β-fructosidase activity in this steady-state culture of *K. marxianus*. However, continuous growth of *S. cerevisiae* was performed at a dilution rate of 0.18 h^{-1}. Hence, a steady-state culture of *K. marxianus* growing at $D = 0.18$ h^{-1} was also monitored. During 15 volume changes, the total inulinase activity also remained constant at 36.8 ± 1.4 U mg of cells (dry weight)$^{-1}$. The percentage of inulinase present as supernatant inulinase was always between 46 and 57%.

DISCUSSION

Invertase production and cell division cycle. The activity of invertase in synchronous cultures of *S. cerevisiae* CBS 8066 showed a cyclic pattern that coincided with the budding cycle of the yeast. The synthesis of invertase was lowest during the single-cell phase. When the percentage of budding cells increased, the production of invertase increased (Fig. 1). This supports the hypotheses that production of extracellular enzymes in yeasts is related to the increased amount of transport vesicles during budding (2, 5, 17) and that de novo-synthesized and de novo-secreted invertase is mainly present at the developing bud (17).

The maximal production of invertase in a synchronous continuous culture of *S. cerevisiae* was about three times lower than in an asynchronous continuous culture of *S. cerevisiae* growing at the same dilution rate. That the residual sugar concentration in the culture liquid and the intracellular hexose-6-phosphate concentration of a synchronous culture were significantly higher than in an asynchronous culture is of interest. Invertase production is subject to catabolite repression (9, 10), and the lower level of invertase in an oscillating culture could be due to the higher glucose and hexose-6-phosphate concentrations. In synchronous chemostat cultures, the residual sugar concentration did not oscillate, indicating that cyclic changes in invertase production do not result from changes in residual glucose. A candidate to regulate invertase synthesis is the intracellular hexose-6-phosphate concentration (1, 20). Indeed, the hexose-6-phosphate concentration showed oscillations related to the budding cycle. After the intracellular level of glucose-6-phosphate was approximately 26 mg/g, the decay in invertase levels coincided with a supplementary increase in intracellular hexose-6-phosphate (Fig. 1B and C). The decrease in total invertase activity during cell scission and the single-cell phase could not be explained only by cessation of invertase synthesis and washout. During the single-cell phase a decay of invertase by either inactivation or denaturation of the enzyme must have occurred. Remarkably, the onset of the decay stage coincided with the moment that the hexose-6-phosphate concentration had reached its maximal value.

Invertase localization and cell cycle. Only the cell-bound invertase showed cyclic changes in invertase level. It was expected that during budding more invertase would be released into the culture fluid. However, the invertase present in the culture fluid (supernatant invertase) remained constant throughout the oscillations of the culture and thus throughout the cell cycle (Fig. 1C). The cell extracts contained both the intracellular invertase and cell wall-retained invertase. In view of the fast solubilization of invertase during cell disruption, it is likely that these changes in invertase mainly concern cell wall-retained enzyme. Moreover, the intracellular invertase accounts for only 5% of the total invertase activity (5, 10). It is apparent that during budding nearly all of the newly synthesized invertase remains in the cell wall. Only a small part is released into the culture fluid, thus compensating for the washout of supernatant enzyme. This release is a continuous process and is not dependent on the cell cycle. Therefore, release of the enzyme in the culture fluid is probably caused by culture conditions such as ionic strength of the medium or shear force; release is not regulated by the yeast cell. This view is supported by the findings that supernatant invertase is a dimer and cell wall-retained invertase is an octamer and that dissociation of an octamer into dimers is affected by ionic strength of the medium and by shear force (5).

In spite of a constant invertase level in the culture fluid during steady-state cultivation, the percentage of invertase present in this fraction was between 40 and 49%. Contrary to our results, the invertase of *S. cerevisiae* has been reported to be barely present (<10%) in the culture fluid (5, 10, 11). However, these reports are mostly based on batch culture studies using complex media. The shear force, a condition that could affect the release of invertase from the cell wall (5), is much less in culture flasks than in chemostats. No continuous culture studies on both production and distribution of invertase in *S. cerevisiae* have been carried out. McMurrough and Rose (10) established the invertase production in continuous cultures of *S. cerevisiae* but did not determine the invertase activity in the culture fluid.

Inulinase production and localization in asynchronous cultures of *K. marxianus*. In contrast to the production of invertase in asynchronous steady-state cultures of *S. cerevisiae* CBS 8066, there were no significant changes in inulinase production during steady state in cultures of *K. marxianus*. Recently, we reported on the production and distribution of inulinase in continuous cultures of *K. marxianus* CBS 6556 (14). A rather broad variation in the distri-

bution of inulinase over supernatant and cell wall fraction was found between cultures grown at different dilution rates (14). A closer look into the distribution of inulinase during steady states at $D = 0.1\ h^{-1}$ and $D = 0.18\ h^{-1}$ reveals that these variations are not significant.

Applied aspects. The results presented above clearly show that production of the extracellular invertase is dependent on the budding cycle of *S. cerevisiae*. Since this organism easily synchronizes in aerobic sugar-limited cultures, it is clear that large variations in enzyme production may occur. This phenomenon may also hold for production of heterologous proteins by *S. cerevisiae*. The occurrence of oscillations seems to be peculiar to Crabtree-positive yeasts (1, 18) such as *S. cerevisiae*. The use of Crabtree-negative yeasts, such as *K. marxianus*, for production of heterologous, glycosylated proteins may offer the advantage of an absence of oscillations and a constant protein production level.

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