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Comparative Study on the Production of Guar α-Galactosidase by *Saccharomyces cerevisiae* SU50B and *Hansenula polymorpha* 8/2 in Continuous Cultures

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***Saccharomyces cerevisiae* SU50B and *Hansenula polymorpha* 8/2, both carrying a multicopy integrated guar α-galactosidase, have been cultivated in continuous cultures, using various mixtures of carbon sources and cultivation conditions. Both *S. cerevisiae* SU50B and *H. polymorpha* 8/2 are stable and produce high levels of extracellular α-galactosidase in continuous cultures for more than 500 h. For these expression systems the strong inducible promoter systems GAL7 and methanol oxidase, respectively, were used. The induction of α-galactosidase synthesis by galactose in SU50B is limited by the low galactose uptake. Apart from that, at high dilution rates, the glucose repression is substantial, and a maximal expression level of 28.6 mg of extracellular α-galactosidase · g (dry weight) of biomass^{-1} could be obtained. In *H. polymorpha*, the induction of α-galactosidase synthesis, in addition to methanol oxidase synthesis using formaldehyde, is very effective up to 42 mg of extracellular α-galactosidase · g (dry weight) of biomass^{-1}. Productivities in terms of specific production rate enable a good comparison with those of other heterologous expression systems in the literature. The productivities found with *S. cerevisiae* SU50B and *H. polymorpha*, 3.25 and 5.5 mg of α-galactosidase · g of biomass^{-1} · liter^{-1} · h^{-1}, respectively, rank among the highest reported in the literature. Enzyme production and secretion in *H. polymorpha* are more complex. A two-peaked optimum is found in enzyme production. No clear explanation of this phenomenon can be given.**

The enzyme α-galactosidase has many potential applications in the modification of galactomannans, e.g., guar gum (16) and galactose-containing oligosaccharides such as raffinose and stachyose. It can remove 1,6-linked α-D-galactopyranosyl groups. Compared with various other α-galactosidases, the α-galactosidase from the plant *Cyamopsis tetragonoloba* (guar) has the special property of being able to work effectively at low water activities and on polygalactans (2). One of the applications of guar α-galactosidase is in the manufacture of improved gelling agents that are based on galactomannans.

In the past few years the α-galactosidase gene from the guar plant has been cloned into *Bacillus subtilis* (18), *Hansenula polymorpha* (5), and *Saccharomyces cerevisiae* (33). In *H. polymorpha* the expression of heterologous genes is under the control of the very strong, inducible methanol oxidase (MOX) promoter (12). MOX under its own promoter may account for 40% of the total soluble proteins in this yeast. The MOX promoter can be induced by methanol, but in continuous cultures it is also induced very efficiently by formaldehyde and formic acid (9). In *S. cerevisiae* the production of α-galactosidase is under the control of the inducible GAL7 promoter. As pointed out by Johnston (10), most of the genes in the catabolism of galactose are under glucose repression and induced by a galactose derivative made by the *GAL3* gene product. Most likely, this inducer is a phosphorylated derivative of galactose (10). The GAL7 promoter is quite strong, and if fully induced, certain *GAL* gene products may account for up to 5% of the soluble proteins in *S. cerevisiae*. For industrial production of food enzymes, the downstream processing costs should be as low as possible. Consequently, secretion of the enzyme into the fermentation medium and the subsequent removal of yeast cells by microfiltration techniques and concentration of the enzyme by ultrafiltration techniques constitute the most attractive production route. Therefore, we placed in front of the α-galactosidase gene the sequence encoding the signal sequence of invertase to ensure secretion of the enzyme into the medium. In shake flask cultures, it has been shown that, in both *H. polymorpha* and *S. cerevisiae*, α-galactosidase is secreted into the medium and folded correctly into an active enzyme.

There has been considerable experience with the fed-batch production of *S. cerevisiae* on inexpensive substrates such as molasses. Recent publications show the potential expression of homo- and heterologous genes located on plasmids in *S. cerevisiae*, using galactose as an inducing substrate in fed-batch fermentations (1, 13). Continuous-culture studies provide more defined information than batch or fed-batch processes on physiological questions such as biomass yields, maximum or critical growth rates, specific substrate or inducer consumption rates, optimal induction levels, and productivity of enzymes. Continuous culturing of *H. polymorpha* has been studied extensively (4, 6). Also, the production of homo- and heterologous proteins by *H. polymorpha* (28) or the closely related *Pichia pastoris* (27) in batch and fed-batch processes has been studied in detail. In this study, the production of the heterologous enzyme α-galactosidase by *S. cerevisiae* and *H. polymorpha* in continuous cultures has been studied in detail.

In earlier publications, we introduced the simple formula $P/V = D \cdot X \cdot E \cdot \mathrm{Se}$ to describe the productivity of homo- and heterologous proteins by microorganisms. This formula

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describes the productivity, P/V (amount of secreted α-galactosidase produced per liter per hour), as a function of the growth rate (D), the biomass (X), the expression rate per biomass (E), and the efficiency of secretion (Se) of α-galactosidase. The results have been used to quantify the above productivity formula.

Since continuous cultures are also the most suitable tool for studying genetic stability under well-defined selective pressure, the genetic stability has been tested as well.

MATERIALS AND METHODS

Strains and genetic constructs. *S. cerevisiae* SU50, YT6-2-1L, and CBS 235.90 have been used in this study (phenotype: MEL^- $HIS4^-$ $LEU2^-$ CIR^0 [14, 33]). Strain SU50 has been transformed with integration plasmid pUR2774. On this plasmid the α-galactosidase gene has been placed under the control of the inducible GAL7 promoter. The integration took place on the rDNA locus (14). The transformant used for this study had 100 to 110 copies of pUR2774 integrated. *H. polymorpha* 8/2 was a transformant derived from strain A16 ($LEU2^-$) and selected by the method of Veale (5, 31). The transformed cell contains three copies of α-galactosidase under control of the MOX promoter integrated in the MOX locus (24).

Media. All media were designed to result in a carbon-limited chemostat. Deproteinized hydrolyzed whey (DHW) was obtained from DMV Campina, Veghel, The Netherlands. After ultrafiltration (molecular weight cutoff, <30,000) and dilution, the DHW solution contained 116 g of glucose and 83 g of galactose $liter^{-1}$. The media used were derived from those reported by Egli (4) and Giuseppin et al. (8). Medium 1 was composed of the following (in grams $liter^{-1}$): NH_4Cl, 7.6; KH_2PO_4, 2.8; $MgSO_4 \cdot 7H_2O$, 0.6; yeast extract (Difco), 5; glucose, 20; histidine, 0.05; trace metal solution (4), 10; vitamin solution (4), 2. Medium 2 contained the following (in grams $liter^{-1}$): medium 1 with glucose, 5.5; DHW (DMV), 125. Medium 3 was medium 2 concentrated 2.5 times. Medium 4 was composed of the following (in grams $liter^{-1}$): NH_4Cl, 7.6; KH_2PO_4, 4.0; $MgSO_4 \cdot 7H_2O$, 0.6; glucose, 20; galactose, 10 (set to 0 to 20 g $liter^{-1}$); histidine, 0.2; trace metal solution (4), 10; vitamin solution (4), 1. Medium 5 was made up of medium 1 with galactose, 10; histidine, 0.1; and leucine set to 0.2 to 0.4 g $liter^{-1}$. The media were autoclaved for 20 min at 121°C. The vitamin solutions were filter sterilized. *H. polymorpha* was grown on medium 1 without histidine.

The galactose/glucose ratio was set at 10:20 by adding glucose.

Fermentation equipment. Continuous cultivations were carried out in 1-liter fermentors with a magnetic bottom drive. Most studies were carried out in two parallel fermentors. The working volume was 0.85 to 1.0 liter. The pH was controlled with 10% NH_4OH at 5.0, and the temperature was maintained at 30.0°C. The dissolved oxygen tension was monitored and was kept above 25% air saturation by manual adjustment of the air flow. Foaming was controlled by using silicone oil Rhodorsil 426R.

Assays. Fermentor inlet and exhaust gases were analyzed automatically with a mass spectrometer (MM8-80; Fisons VG Instruments, Middlewich, United Kingdom) for O_2, CO_2, ethanol, and N_2 (for volume corrections). Generally, steady states were obtained after five fermentor dilutions. Subsequently, two consecutive samples were taken in duplicate and analyzed. The mean values are given in the tables and figures. Various dilution rates were duplicated in parallel fermentors to test reproducibility.

Biomass dry weight was determined by drying the twice-washed samples for 16 h at 110°C.

α-Galactosidase was determined by the protocol of Overbeeke et al. (19). Specific activities of 100 and 38 U · mg of $protein^{-1}$, respectively, were determined for α-galactosidase for SU50B and *H. polymorpha* 8/2, using the method of Fellinger et al. (5). One unit liberates 1 μmole of *p*-nitrophenol per minute from *p*-nitrophenyl-α-D-galactoside under the assay conditions.

MOX activity was determined by the method of van Dijken et al. (30).

Metabolites were determined by using a high-performance liquid chromatograph (Aminex HPX; Bio-Rad) with a refractometer; 0.01 M H_2SO_4 was used as the eluent. The column temperature was 60°C.

Cell lysates were obtained with twice-washed cells (0.1 M potassium phosphate buffer, pH 7.0). A 0.5-ml suspension with an optical density at 610 nm of 20 to 30 was put in a small glass tube (75 by 12 mm). Glass beads (diameter, 0.5 mm) were added to 2 mm above the liquid surface. The mixture was shaken on a Vortex vibrating table for 90 s, after which 0.5 ml of potassium phosphate buffer was added and mixing was continued for three 60-s periods. Up to 64 samples of lysates were also obtained in a single run, using Eppendorf cups (1.5 ml). To 1 ml of a twice-washed cell suspension of optical density (610 nm) of 20 to 30 was added 0.5 g of glass beads. The cups were placed in a multirack (type V/X2E) that can hold 64 cups. This rack was shaken on a Vibrax shaker (type VXR; IKA Labor Technik) for 3 min. Both methods resulted in an effective cell lysis (>90%) as judged by microscopic examination.

The protein content was determined by the method of Lowry et al. (15), with bovine serum albumin as the standard.

RESULTS

***S. cerevisiae* SU50B in continuous cultures: characteristics of the *S. cerevisiae* SU50 parent strain.** The leucine-requiring parent strain SU50 was studied in continuous cultures to determine the main growth characteristics, in particular, glucose repression, the related decrease in growth yield, and the effects of the leucine dependency of this strain. This would give more information on the physiological background and on the consequences of complementing *LEU2* by the multicopy integration plasmid.

After extensive adaptation to the chemically defined growth medium (medium 5 with 400 mg of leucine and 100 mg of histidine · $liter^{-1}$), a stable culture with 6.7 g of biomass · $liter^{-1}$ was obtained at a dilution rate of 0.05 h^{-1}. In spite of the low biomass, the complete consumption of glucose, and the high oxygen tension in the medium, large amounts of ethanol were produced, resulting in an ethanol concentration of 8.56 g · $liter^{-1}$. Galactose was consumed partly. The residual galactose concentration was 5.5 g · $liter^{-1}$.

Not including leucine as a carbon source, the carbon balance is complete up to 99.6%. No by-products such as acetate and pyruvate are formed. The carbon in the feed was 0.05 mol · $liter^{-1}$ · h^{-1} in glucose and galactose.

A high amount of leucine in the feed did not improve the biomass yield. Also, low dilution rates (<0.025 h^{-1}) were ineffective in reducing ethanol formation. Therefore, growth of *S. cerevisiae* under these conditions can be described as

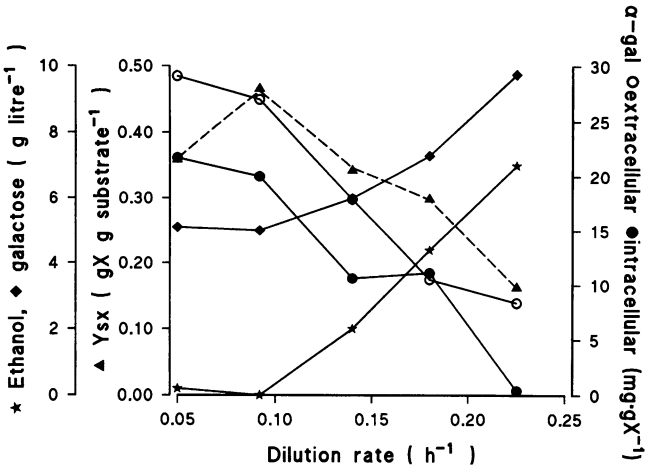

FIG. 1. *S. cerevisiae* SU50B in continuous cultures: effect of dilution rate. Medium 4 with galactose/glucose at 20:10 was used. α-gal, α-galactosidase; X, biomass.

limited by the glucose concentration in the media and the leucine uptake rate.

Continuous cultivation of transformed *S. cerevisiae* SU50B in chemically defined media. *S. cerevisiae* SU50 was transformed as described by Verbakel (33), resulting in *S. cerevisiae* SU50B. This strain was cultivated continuously in a chemically defined medium. Several transformants were tested, using a range of galactose/glucose ratios that allow economic production of α-galactosidase. The data obtained from one selected transformant are given.

The best results were obtained by using a fixed galactose/glucose ratio of 1:2. Figure 1 shows the effects of the dilution rate. The biomass formation drops dramatically above D = 0.1 h^{-1}. This is correlated with the formation of ethanol and a reduced consumption of galactose. The glucose levels were below 0.15 mM up to a dilution rate of 0.225 h^{-1}. The glucose repression will be low under all conditions. Nevertheless, ethanol is formed, which reflects a strong Crabtree-like effect in this yeast strain and which may be related to the low leucine and oxygen uptake capacities.

Metabolic fluxes. The fluxes of O_2 and CO_2 and metabolites are given in Fig. 2. It can be seen that this strain reaches an oxygen uptake plateau at 4.1 mmol of O_2 · g of biomass^{-1} · h^{-1}. This value is lower than that usually found for baker's yeast (6 to 12 mmol of O_2 · g of biomass^{-1} · h^{-1} [20, 21]). According to the limited oxidation capacity hypothesis of Sonnleitner et al. (26), the low oxygen uptake rate may be the reason for the low critical dilution rate of 0.1 h^{-1} at which ethanol formation occurs. Analysis of the fluxes also shows the increased glucose consumption which parallels the formation of both ethanol and acetate. Carbon recovery was higher than 95%.

Enzyme secretion. The production of α-galactosidase varies as a function of the growth rate (Fig. 1). Figure 3 shows the specific enzyme production rate, which facilitates the comparison at various dilution rates. The decrease at 0.225 h^{-1} is dramatic. The ratio between intra- and extracellular α-galactosidase is strongly dependent on the growth rate. Whereas at a low growth rate (D = 0.05 h^{-1}) about 50% of the α-galactosidase was secreted, the secretion increased to >75% at a dilution rate of 0.1 h^{-1}. A further increase in the growth rate decreased the efficiency of the secretion process, probably because of energy shortage.

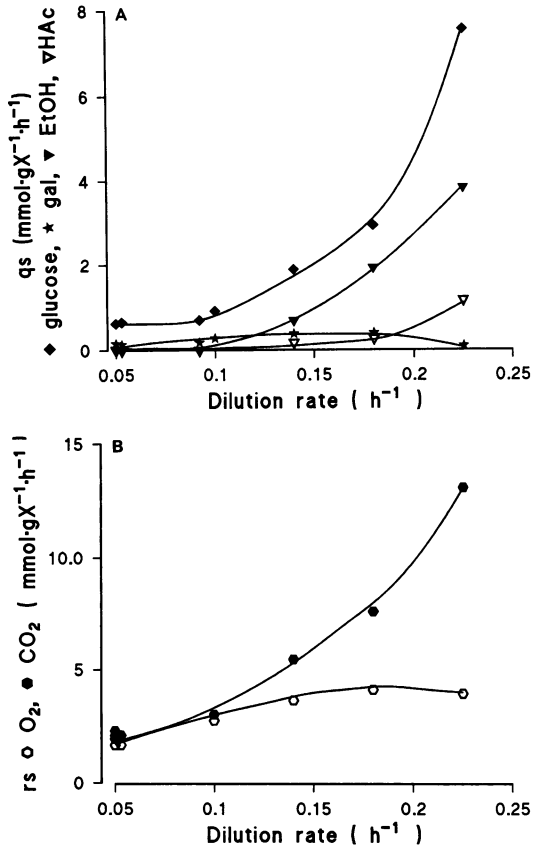

FIG. 2. *S. cerevisiae* SU50B in continuous culture. (A) Effect of dilution rate on specific production and consumption rates (q · s). EtOH, ethanol; X, biomass. (B) Effect of dilution rate on specific CO_2 production and O_2 consumption rates (rs).

Effect of galactose/glucose ratio. Three galactose/glucose ratios were tested at a D of 0.1 h^{-1}, using medium 4 with 20 g of glucose liter^{-1}. The steady-state values given in Table 1 show that a galactose/glucose ratio of 0.5 results in the highest production of α-galactosidase. The biomass formation is slightly lower because of less contribution from

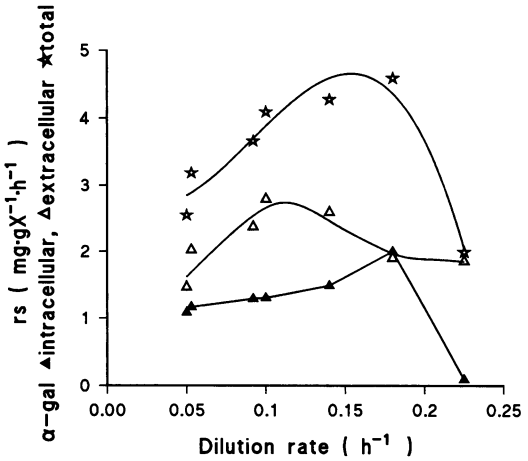

FIG. 3. *S. cerevisiae* SU50B-specific α-galactosidase production (rs). X, biomass.

TABLE 1. Effect of glucose/galactose ratio on production of α-galactosidase[a]

Ratio, galactose/glucose	α-Galactosidase (mg · liter^{-1})[b]	Galactose (g · liter^{-1})[c]	Ethanol (g · liter^{-1})[c]	Biomass (g · liter^{-1})[d]	α-Galactosidase per biomass (mg · g of biomass^{-1})	Galactose consumption (g · h^{-1} · g of biomass^{-1})
0/20	20	0	0.34	10.4	1.9	0
10/20	403[e]	0.03	0.26	14.1	28.7[e]	0.07
15/20	300	4.0	0.18	15.9	18.8	0.069
20/20	330	7.0	0.15	16.0	20.6	0.064

[a] The glucose concentration in all steady states was below the detection limit of 0.025 g · liter^{-1}.
[b] Standard deviation, 5% absolute.
[c] Standard deviation, ±0.05 g · liter^{-1}.
[d] Standard deviation, ±0.1 g · liter^{-1}.
[e] Highest α-galactosidase production significant at $P < 0.05$.

galactose. The biomass yield and derepression of α-galactosidase production on glucose as the sole carbon source are given as references. The yield of the strain, 0.52 g of biomass · g of glucose^{-1}, is relatively high. The derepression yields a level of α-galactosidase of only 5% of the induced level. The observed galactose consumption rates were about 0.07 g · g of biomass^{-1} · h^{-1} over the whole D range tested, which is close to the maximal uptake rates in batch cultures grown on galactose. This strongly indicates that all of the galactose permease molecules are already saturated with galactose.

Continuous cultivation of the transformed *S. cerevisiae* SU50B strain in commercial media. DHW as the carbon and vitamin source was tested as a potential commercial medium for the production of α-galactosidase by *S. cerevisiae*. Commercially attractive dilution rates of 0.05 and 0.1 h^{-1} were chosen.

Table 2 shows the results for low-density cultures. A 2.5-fold increase in carbon source (medium 3) gave a higher yield of α-galactosidase at $D = 0.05$ h^{-1} (significant at $P < 0.05$). At $D = 0.1$ h^{-1}, ethanol formation is pronounced, which consequently lowers the cell yield. The enzyme yield is also lower. The ethanol formation is not due to a limitation in oxygen transfer or ineffective mixing, as judged from oxygen traces at different aeration rates.

***H. polymorpha* 8/2 in continuous cultures: effect of dilution rate in glucose-limited continuous cultures on production of α-galactosidase.** *H. polymorpha* 8/2 was cultivated in glucose-limited continuous cultures at $D = 0.1$ h^{-1}. Under these conditions, glucose-repressible genes are in the derepressed state. This derepressed state resulted in a MOX activity of about 0.8 U · mg of protein^{-1} (Fig. 4). The MOX level in transformed cells is higher than that in the wild-type strain, which shows an activity of 0.4 U · mg of protein^{-1} (4, 7–9).

No by-products are found, and the C balance is complete within 94%. The maximum yield is 0.55 g of biomass · g of glucose^{-1}. At lower dilution rates, the maintenance energy component in the energy consumption becomes relatively higher, as indicated by the lower yield (Y_{SX} = 0.46 g of biomass · g of glucose^{-1}, where Y_{SX} is the biomass yield on substrate).

Production of α-galactosidase during growth at various dilution rates on media with formaldehyde/glucose as feed. The production of α-galactosidase was tested with formaldehyde, a good inducer of the MOX promoter (6, 9). The effect of the formaldehyde/glucose ratio has been reported previously (6, 9) for wild-type *H. polymorpha*. On the basis of former experiments, formaldehyde/glucose feeds with molar ratios of 0, 0.5, and 1.0 were tested. In medium with a formaldehyde/glucose ratio of 1.0, the yield on biomass (0.52 g of biomass · g of substrate^{-1}) is high up to a dilution rate of 0.2 h^{-1}. Above this value, significant amounts of formaldehyde could be detected in the medium and respiration decreased.

The yield on biomass for all cultures decreased from 0.52 g of biomass · g of substrate^{-1} for high dilution rates to 0.47 g of biomass · g of substrate^{-1} for low dilution rates.

The best productivities were obtained with a formaldehyde/glucose ratio of 0.5. Although this ratio was suboptimal with the wild-type strain (9), growth in this medium resulted in high expressions of both MOX and α-galactosidase. Figure 5A and B shows the activities of the enzymes. Under these conditions, the MOX activity is more than twice that obtained during growth in formaldehyde/glucose at a ratio of 1.0. The expression of enzymes under control of the MOX promoter has a two-peaked D profile, comparable to the formaldehyde/glucose ratio of 1.0. The biomass yield is not significantly lower than that obtained with the formaldehyde/glucose ratio of 1.0. This may indicate that formaldehyde is

TABLE 2. Steady states of SU50B on DHW[a]

D (h^{-1})	OD_{610} (AU)[b]	α-Galactosidase[c]		Biomass (g · liter^{-1})[d]	Ethanol (g · liter^{-1})	Glucose (g · liter^{-1})	Galactose (g · liter^{-1})	rO_2 (mmol · liter^{-1} · h^{-1})	rCO_2 (mmol · liter^{-1} · h^{-1})	Y_{SX} (g of biomass · g of substrate^{-1})
		Extracellular (mg · liter^{-1}	Intracellular (mg · g of biomass^{-1})							
0.05	9.31	460	5.4	9.36	0.66	0.6	6.33	19.9	21.0	0.41
0.10	13.0	360	—[e]	11.1	0.26	0.5	7.65	47.6	47.5	0.51
0.05	23.0	1,100	30.8	24.3	0.8	0	11.4	53.1	53.2	0.38
0.10	17.5	440	13.0	16.3	7.0	0	15.7	16.7	17.8	0.27

[a] The standard deviation was <0.2 g · liter^{-1} unless otherwise specified.
[b] OD_{610}, optical density at 610 nm.
[c] Standard deviation, 5% absolute.
[d] Standard deviation, <0.1 g · liter^{-1}.
[e] —, not determined.

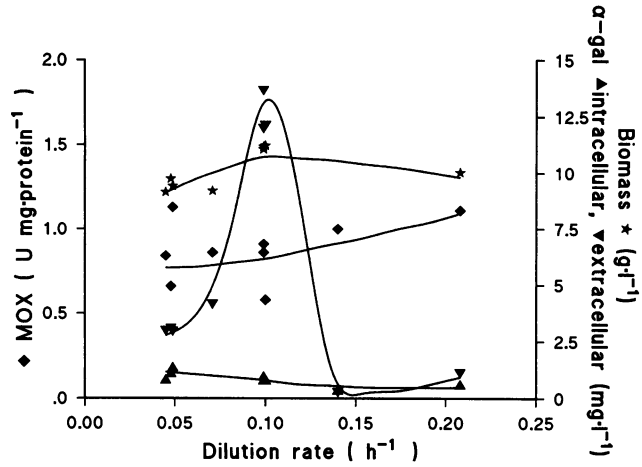

FIG. 4. *H. polymorpha* 8/2 in continuous cultures. Medium 1 with 20 g of glucose · liter^{-1} was used.

dissimilated ineffectively as an additional energy source also at this higher ratio. The respiration characteristics at the low ratio of 0.5 were similar to those found for the formaldehyde/glucose ratio of 1.0. Yield coefficients of 25.0 g of biomass · mol of O_2^{-1} and 27.5 g of biomass · mol of CO_2^{-1} were determined. However, at high dilution rates (0.2 h^{-1}) no decrease in respiration was found. A formaldehyde ratio of 0.5 will still allow effective induction of the dissimilatory pathway. The maximum uptake flux of formaldehyde for wild-type *H. polymorpha* is about 2 mmol · g of biomass^{-1} · h^{-1} (9). This maximum value is reached in these cultures at dilution rates of 0.19 and 0.38 h^{-1} for formaldehyde/glucose ratios of 1.0 and 0.5, respectively.

MOX synthesis. MOX expression shows two optima. The highest optimum is about 80% of that in the untransformed strain (Fig. 5B). The first optimum is at D = 0.08 h^{-1}. The second, and lower, one is at 0.17 h^{-1}. Assuming a specific activity of 25 U · mg of protein^{-1}, this would imply that 45% of the total intracellular protein is MOX. Unexpectedly, an increased formaldehyde/glucose ratio of 1.0 resulted in a constant low activity, 3 to 4 U · mg of protein^{-1} (data not shown).

α-Galactosidase expression is given in Fig. 5A. A two-peaked D profile parallels the MOX expression. It is remarkable that a second, lower optimum occurred. This is a statistically significant difference ($P < 0.05$). An intracellular level of α-galactosidase activity of 6.6% at D = 0.08 h^{-1} is slightly lower than the formaldehyde/glucose ratio of 1.0. A higher extracellular level of 426 mg · liter^{-1} at D = 0.08 h^{-1} is found. Judging from the electrophoresis gels and the total extracellular protein, more than 87% of the extracellular protein is α-galactosidase. The total α-galactosidase produced represents 16.5% of the total amount of protein produced by *H. polymorpha* 8/2.

The calculated specific enzyme fluxes show that, in the mixture with a formaldehyde/glucose ratio of 0.5, the formation rate of α-galactosidase is 5.5 mg of α-galactosidase · g of biomass^{-1} · h^{-1} (Fig. 6A and B). The fluxes of both intra- and extracellular α-galactosidase are highly correlated. Only at high dilution rates does the secretion rate drop. This is not related to the accumulation of formaldehyde. The formaldehyde flux will reach the maximal level at higher dilution rates of 0.38 h^{-1}.

Ethanol formation. Low oxygen levels, below 5% air saturation, resulted in the formation of ethanol. It is known that ethanol is a potent repressor of MOX synthesis (3). Even low levels of ethanol give a strong repression of MOX and α-galactosidase synthesis. Oxygen limitation in a glucose-limited continuous culture resulted in the production of 1.8 g of ethanol · liter^{-1}, with a drop in α-galactosidase and MOX expression from 12.2 mg · liter^{-1} and 0.58 U · mg of protein^{-1} to 0.24 mg · liter^{-1} and 0.17 U · mg of protein^{-1}, respectively.

DISCUSSION

Fermentation behavior. The formation of ethanol at high growth rates is a known limitation in baker's yeast biomass production. This phenomenon is characterized in continuous cultures by a critical dilution rate above which ethanol

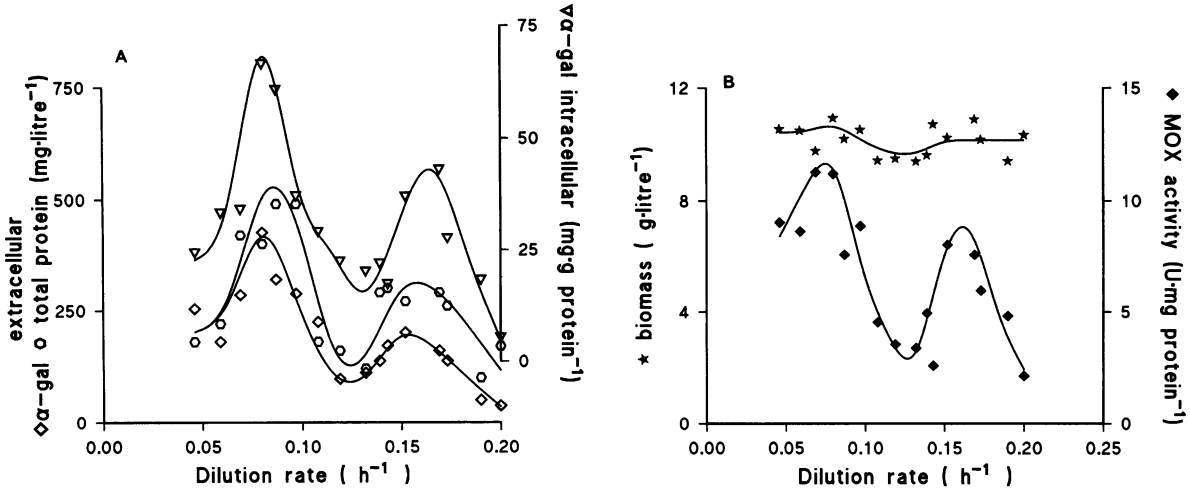

FIG. 5. *H. polymorpha* 8/2 in continuous cultures. (A) Specific enzyme fluxes; intra- and extracellular and total α-galactosidase (milligrams of α-galactosidase · gram of biomass^{-1} · hour^{-1}). (B) Total protein and MOX in milligrams · gram (dry weight) of biomass^{-1} · hour^{-1}. Medium 1 with a ratio of formaldehyde/glucose of 0.5 was used.

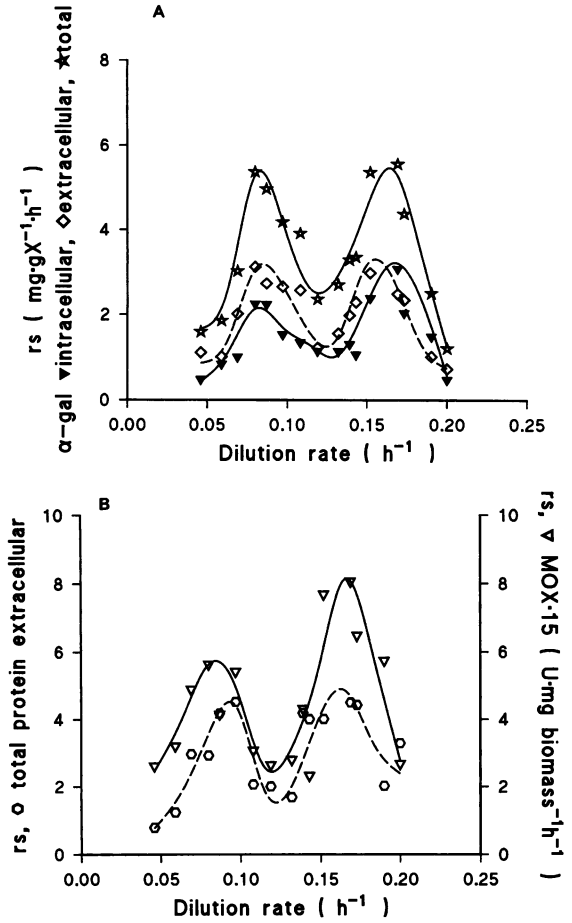

FIG. 6. Enzyme fluxes in continuous cultures of *H. polymorpha* 8/2. (A) Specific enzyme fluxes; intra- and extracellular and total α-galactosidase (milligrams of α-galactosidase · gram of biomass^{-1} · hour^{-1}). (B) Total protein and MOX in milligrams · gram of biomass^{-1} · hour^{-1}. Medium 1 with a ratio of formaldehyde/glucose of 0.5 was used. rs, Specific rate of product formation.

formation occurs. The transformed strain has a critical dilution rate of 0.1 h^{-1}, which is higher than that of the untransformed parent strain SU50. This is still low compared with values of 0.2 to 0.36 h^{-1} with wild-type *S. cerevisiae* strains for both haploid and polyploid production (21, 24). Many haploid laboratory strains used for expression of genes show a low critical dilution rate. Similar high ethanol formation rates at low dilution rates were found in continuous cultures by van der Aar (29) for another haploid *S. cerevisiae* host strain.

The haploid strains of *S. cerevisiae* are clearly different from those of *S. cerevisiae* CBS 8066, as reported by Postma et al. (21). Apart from the low growth rates, they found acetate formation at lower dilution rates than ethanol formation. In contrast to *S. cerevisiae*, *H. polymorpha* showed constant high biomass yields without significant by-product formation over the dilution range tested.

Stability and variability. The selected transformants were extremely stable and gave reproducible results in various parallel experiments. Experiment runs regularly took 500 to 1,000 h without a decrease in α-galactosidase production or gene copy number (data not shown).

Induction and inducer consumption. The induction of α-galactosidase synthesis with galactose in *S. cerevisiae* SU50B is effective despite the low galactose uptake rate. However, at high dilution rates galactose uptake is reduced by glucose repression (23). This drawback of low galactose uptake was overcome in plasmid-based expression systems by using strains with improved galactose induction and less glucose repression (1, 13).

In *H. polymorpha*, the induction of α-galactosidase synthesis with formaldehyde, in addition to MOX synthesis, is very effective. The MOX level in the transformed cell is higher than that in the wild-type strain, which shows an activity of 0.4 U · mg of protein^{-1} (4, 7–9). Under induced conditions, 8 U · mg of protein^{-1} is found. The parent strain A16 produces more MOX under similar conditions, 14.3 U · mg of protein^{-1}, which indicates a negative effect of α-galactosidase expression on MOX expression. The MOX promoter–invertase signal–α-galactosidase sequence is present at three copies, and it is likely that these promoter sequences compete with the original MOX promoter for the activator protein. This may be due to a twofold dilution effect of the repressor proteins, although the existence of specific glucose repressor molecules in *H. polymorpha* has not been demonstrated. However, in *S. cerevisiae* it was found that at least three transcription regulatory proteins, TUP1, SSN6, and MIG1, are involved in specific glucose repression (11). Since even in higher eukaryotes transcriptor regulators are found which are similar to those in yeasts, for example, MIG1 and the mammalian early growth response protein (17), it is not unlikely that in *H. polymorpha* glucose-repressible genes are regulated by similar transcription factors. MOX activity in strain 8/2 is not repressed at high dilution rates as reported for the wild-type strain, which is in agreement with the repressor dilution hypothesis. Indeed, we have found that in the MOX promoter a region is present that complies with the glucose repression sequence, which is recognized by MIG1 in *S. cerevisiae*.

Repression. In batch cultures of *S. cerevisiae* galactose uptake is inhibited by glucose (10). In glucose-limited continuous cultures, a derepression of galactose uptake was determined for an industrial strain of *S. cerevisiae* (25), and derepression is also expected in strain SU50B. The *GAL* genes should be induced under the conditions tested. However, the capacity of galactose permease (encoded by *GAL2*) seems to be too limited for a complete uptake of galactose at higher dilution rates. The galactose uptake fluxes are low, 0.4 mmol of galactose · g of biomass^{-1} · h^{-1}, which is much lower than that for industrial strains with an uptake rate of >2.8 mmol of galactose · g of biomass^{-1} · h^{-1} (25). The low uptake rate of galactose at high growth rates may result in a low intracellular concentration of the inducer of the *GAL* genes and therefore in poor induction (10) since the inducer of *GAL* genes is a product made from galactose by the *GAL3* gene product. The low uptake rate is, in effect, an advantage at low growth rates and for fed-batch fermentations, as it reduces the costs of galactose needed for the process.

Secretion. The secretion in both systems is efficient (41 to 89%) but depends strongly on the dilution rate. In *S. cerevisiae* the secretion decreased at high dilution rates, with an optimum at 0.1 h^{-1}. The specific extracellular α-galactosidase production flux varied only slightly.

The enzyme production and secretion in *H. polymorpha* is more complex. The production depends strongly on the transformant used, but in all cases a two-peaked optimum is found in the production. In *H. polymorpha*, low levels of α-galactosidase synthesized under derepressed (noninduced)

TABLE 3. Productivities of continuous cultures[a]

Strain	Conditions[b]	Biomass (g · liter^{-1})	D (h^{-1})	$(P/V)/[X]$ (mg of α-gal · g of X^{-1} · h^{-1})	Yield (mg of α-gal · liter^{-1})	Yield on S (mg of α-gal · g of S^{-1})	Expression (mg of α-gal · g of X^{-1})	Secretion (%)
S. cerevisiae SU50B	MM	14.1	0.1–0.14	2.9 (4.7)[c]	403 (606)	13.4 (22)	29 (43)	62
S. cerevisiae SU50B	DHW; high [*X*]	24.3	0.05	2.4 (3.8)	1,100 (1,849)	17 (29)	45 (76)	59
S. cerevisiae SU50B	DHW; high [*X*]	16.3	0.1	2.7 (4.0)	440 (651)	7.3 (11)	27 (40)	68
H. polymorpha 8/2	MM; Fa/G ratio, 1	10.9	0.08	2.8 (3.5)	427 (479)	21 (24)	39 (44)	89
H. polymorpha 8/2	MM; Fa/G ratio 1	10.2	0.12	2.5 (4.0)	215 (286)	11 (14)	21 (28)	75
H. polymorpha 8/2	MM; Fa/G ratio, 0.5	10.9	0.08	3.1 (5.4)[c]	426 (583)	21 (29)	39 (53)	73
H. polymorpha 8/2	MM; Fa/G ratio, 0.5	10.9	0.17	3.0 (5.5)[c]	159 (262)	8 (13)	15 (24)	61

[a] The specific fermentor productivity can be described as follows: $P/V = D \cdot [X] \cdot E \cdot \mathrm{Se} \cdot \mathrm{St}$, where X is biomass, E is expression, Se is secretion, and St is stability. Values in parentheses include α-galactosidase activity determined both intra- and extracellularly. α-gal, α-galactosidase; S, substrate.

[b] MM, minimal medium; Fa, formaldehyde; G, glucose.

[c] Significantly higher than the other conditions ($P < 0.05$).

conditions is secreted completely. Secretion under induced conditions is more complex. The production rates of α-galactosidase and MOX in *H. polymorpha* 8/2 show a remarkable characteristic. The results in Fig. 6A and B show two maxima in the synthesis of both the intra- and extracellular forms of α-galactosidase. The total synthesis of >5.8 mg of α-galactosidase · g of biomass^{-1} · h^{-1} is considerable. This is half the maximal rate of MOX synthesis in the wild-type strain. The rate of MOX synthesis (Fig. 6B) also shows a two-peaked profile, which suggests that induction or biosynthesis rather than secretion determines production rate. The total production rate of extracellular protein correlates well with the α-galactosidase production. More than 5 to 5.5 mg of protein · g of biomass^{-1} · h^{-1} was produced, of which more than 80% is α-galactosidase. At low dilution rates the extracellular protein consisted of more than 85% α-galactosidase. No clear explanation of this phenomenon can be given. It may be related to the high flux of formaldehyde in the cell at high dilution rates, which relieves glucose repression. All formaldehyde ratios and transformants tested showed a two-peaked optimum, which rules out any toxic effects of formaldehyde at high dilution rates near the maximal formaldehyde conversion rate.

Specific enzyme production rates. Productivities in terms of specific production rate compared well with those of other heterologous expression systems in the literature. The summary in reference 32 shows that the productivities of both *S. cerevisiae* and *H. polymorpha* are better than those of the other systems tested on the basis of, e.g., *Pichia* sp., which yields 2.9 mg of product · g of biomass^{-1} · h^{-1}. The absolute yield may be higher, but the reported processing times are much longer and biomass concentrations are much higher.

Productivity of continuous cultures. α-Galactosidase is the main protein product in cultures of both *H. polymorpha* and *S. cerevisiae*. Only small amounts of other proteins could be observed. The productivity of continuous cultures was relatively high compared with that of fed-batch cultivation once the culture was in the steady state.

The productivity per amount of biomass can be used to compare the cultures. Table 3 clearly shows the high productivities of both organisms compared with other reported expressions of heterologous genes in high-density fed-batch cultures. Sreekrishna et al. found 1 to 2.9 mg of product · g of biomass^{-1} · liter^{-1} · h^{-1} (27). The growth rate dependence of the specific enzyme production rate shows the need to study the effect of the dilution rate for optimization.

Productivity in terms of P/V depends on the terms $D \cdot [X] \cdot E \cdot \mathrm{Se} \cdot \mathrm{St}$ (see above). The range of D is the same for both organisms, the optimal D value being around 0.1 h^{-1}. The expression level for the total amount of α-galactosidase found is slightly higher for *S. cerevisiae* SU50B. This advantage is counterbalanced by a higher secretion percentage for *H. polymorpha* 8/2 versus *S. cerevisiae*. The term product stability (St) cannot be quantified here. However, extracellular α-galactosidase was very stable during cultivation and storage. The stability of intracellular α-galactosidase is likely to be lower in *H. polymorpha* 8/2 as determined by gel electrophoresis (data not shown).

Large-scale production aspects. *S. cerevisiae* SU50B is a highly productive yeast strain under conditions in which ethanol formation has no negative effects on growth. *S. cerevisiae* SU50B can be considered a GRAS organism, and consequently this strain may be considered a suitable producer of heterologous enzymes for food applications.

The use of high-cell-density cultivation lowers the specific productivity in cultures of *S. cerevisiae* SU50B because of the Crabtree effect. This can be seen in media with high levels of DHW (Table 2). This effect is more pronounced in the case of insufficient micromixing in the culture. Furthermore, even relatively small ethanol fluxes will lead to high ethanol levels in the culture. From other experiments it is known that this strain is sensitive to moderate ethanol concentrations of 10 g · liter^{-1}.

On the other hand, *H. polymorpha* cannot be used for food applications at this time, as a process based on its use has not yet received the GRAS (generally regarded as safe) status. Once this strain has been cleared, it will become attractive, as it has a higher productivity and enzyme yield than *S. cerevisiae* SU50B. Higher cell densities do not suffer from the Crabtree effects, as this strain is Crabtree negative. This was verified in other studies (data not shown). It should be noted that in large-scale cultivation the effects of low oxygen levels may be seen. Then (local) ethanol formation will lead to a repression of the MOX-promoted expression. In this respect *H. polymorpha* presents problems similar to those with *S. cerevisiae* because of ethanol formation. The use of formaldehyde may present safety issues. However, no residual formaldehyde is found up to the maximal uptake rate, which makes this process safe and robust.

The information on induction conditions is used to set up fed-batch fermentations with *H. polymorpha* or *S. cerevisiae* as the host organism. The productivity may be increased by using high cell densities. A five- to tenfold increase in biomass with a comparable expression level may be obtained, which enables efficient production of guar α-galactosidase for commercial applications.

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