Enzyme Pattern and Aerobic Growth of *Saccharomyces cerevisiae* Under Various Degrees of Glucose Limitation

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The enzyme pattern of *Saccharomyces cerevisiae* was followed during batch growth and in continuous culture in a synthetic medium limited for glucose under aerobic conditions. Seven enzymes were measured: succinate-cytochrome c oxidoreductase, malate dehydrogenase, nicotinamide adenine dinucleotide-linked glutamate dehydrogenase, malate synthase, isocitrate lyase, aldolase, and nicotinamide adenine dinucleotide phosphate (NADP⁺)-linked glutamate dehydrogenase. During fermentation of glucose and high growth rate (μ) during the first log phase in batch experiments, the first five enzymes (group I) were repressed, and aldolase and NADP⁺-linked glutamate dehydrogenase (group II) were derepressed. During growth on the accumulated ethyl alcohol and lower μ, the group I enzymes were preferentially formed and the other two were repressed. A sequence of derepression of the group I enzymes was found during the shift from glucose to ethyl alcohol metabolism, which can be correlated with a strong increase in the percentage of single (nonbudding) cells in the population. A correlation between the state of cells in the budding cycle and enzyme repression and derepression is suggested. In continuous culture, the enzyme pattern was shown to be related to the growth rate. The group I enzymes were repressed at high growth rates, while the group II enzymes were derepressed. Each enzyme exhibits a different dependence. The enzyme pattern is shown to depend on the rate of substrate consumption as well as on the type of metabolism and to be correlated with the budding cycle. The enzyme pattern is considered to be controlled by changes of intracellular catabolic or metabolic conditions inherent in the division cycle.

Glucose increases the rate of carbon dissimilation via the glycolytic pathway of bakers' yeast, even under aerobic conditions. The phenomenon, called "aerobic fermentation" (11), has been explained by a regulatory mechanism analogous to the "Crabtree effect" (2). This metabolic change to fermentation following the addition of glucose gives rise to a high rate of energy production, although the gain of energy is low. Glucose is rapidly metabolized to ethyl alcohol and CO₂, while the fast energy production allows maximal specific growth rate [μ (max) = 0.42] during a first log phase (17, 21).

It was shown recently (10, 27, 29, 32) that during batch growth of *Saccharomyces cerevisiae* on glucose, the formation of respiratory, tricarboxylic acid cycle and glyoxylate bypass enzymes is inhibited, in a manner analogous to "catabolite repression" (16). Substrates which support growth at a lower specific growth rate, e.g., maltose or galactose [μ (galactose) = 0.25, μ (maltose) = 0.34; Beck, unpublished data], cause less marked inhibition (10, 27, 29). Derepression of the same enzymes was observed in the second growth stage when the culture is growing at the expense of ethyl alcohol accumulated during the first stage. Depending on the composition of the medium, specific growth rates [μ (ethyl alcohol)] between 0.12 and 0.18 were obtained.

By use of the technique of continuous culture (chemostat), different growth rates with populations of *S. cerevisiae* were maintained; these rates could be correlated with actual types of metabo-

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lism under glucose limitation. A distinct relationship was demonstrated between the degree of fermentation and oxidation and the growth rate (20). With increasing fermentative metabolism at growth rates above 0.20, a repression of the specific oxygen uptake has been observed (7, 8).

Correlations between the specific growth rate and the enzyme patterns under glucose limitation in continuous culture of *S. cerevisiae* are presented in this paper. A batch experiment carried out allows a comparison with results reported in literature (10, 27, 29).

**Materials and Methods**

*Organism.* *S. cerevisiae* (bakers' yeast) strain LBG H 1022 was used. For the batch experiment, a 12-hr culture, grown in the same medium, was used as inoculum.

**Growth conditions.** Cells were grown at 30°C in a liquid synthetic medium, NL 10, previously described (20), with glucose as limiting substrate (glucose concentration $S_0 = 9.2$ mg/ml). A bench-scale fermentor (Chemag AG., Männedorf, Switzerland) served as cultivation device (6). During growth in batch as well as in continuous culture, the pH was automatically controlled at 5.5 by the addition of 2 N sodium hydroxide or 2 N phosphoric acid. Aerobic conditions were maintained by an airstream of 0.5 liters per liter of medium per min and by mixing with flat-blade turbines (900 rev/min; maximal oxygen transfer rate, 2,200 ml of O$_2$ per liter per hr). For batch experiments, the complete medium was sterilized in the fermentor vessel; for continuous culture, the glucose-vitamin solution was autoclaved separately from the mineral salts and combined afterwards.

Continuous culture was carried out according to the chemostatic principle (24). The working volume $V$ was kept constant at 3.0 liters. The dilution rate $D$ was varied by changing the medium flow rate $F$ ($D = F/V$). The course of the cultivations was followed by measuring dry weight, glucose, ethyl alcohol concentration, and respiration quotient (RQ). Steady states were generally reached within 12 mean generation times ($g = 0.693/D$) after a change in flow rate $F$ (without intervening disturbances of the cultivation). At steady states, the value for specific growth rate ($\mu$) of the population is identical with the adjusted dilution rate ($D$).

**Analytical methods.** The determinations of dry weight ($X$), glucose ($S$), and ethyl alcohol concentration ($A$) were carried out as previously described (7). The specific glucose uptake rate $Q_8$ was calculated by the equation $Q_8 = (S_0 - S)/X - D$ and is expressed as milligrams of glucose consumed per milligram (dry weight) per hour. Oxygen and carbon dioxide in the outflowing airstream were determined continuously by use of gas analyzers (Hartmann and Braun, Frankfurt, Germany). From these data, the respiration quotient was calculated (Fiechter and von Meyenburg, in press).

**Preparation of cell-free extracts.** A yeast-cell suspension, containing 0.6 to 1.0 g (wet weight), was sampled from the growing cultures (single samples in batch, duplicates in continuous-culture experiments), harvested in a cooled centrifuge at 2,000 $\times$ g, and washed twice with ice-cold 0.067 M Sörensen phosphate buffer, pH 6.5. For disruption, the cell pellet was resuspended in 6 ml of buffer and mixed with 15 ml of glass beads ($D = 0.45$ to 0.50 mm). The mixture was violently agitated by a Vibromix stirrer (Chemag AG., Männedorf, Switzerland) in a 40-ml centrifuge tube for 4 min, which resulted in 95% cell disintegration. The temperature was kept at 2 to 5°C throughout the operation. This method of cell disruption is similar to the one described by Novotny (25). After the separation of the glass beads, the cell debris and the unbroken cells were centrifuged at 2,000 $\times$ g for 10 min. Cell-free extracts were either used immediately or were frozen and kept at $-20$°C without severe loss of enzyme activity. Protein concentration in the extracts was estimated according to the method of Lowry et al. (14).

**Measurement of enzyme activities.** Malate dehydrogenase (EC 1.1.1.37) and fructose diphosphate aldolase (EC 4.1.2.13) activities were determined by "Boehringer test combinations" (C. F. Boehringer & Söhne, Mannheim, Germany); isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) were determined according to the method of Dixon and Kornberg (3), but at pH 6.5 and 7.3, respectively. The two glutamate dehydrogenase activities [nicotinamide adenine dinucleotide (NAD$^+$) and nicotinamide adenine dinucleotide phosphate (NADP$^+$)-linked, EC 1.4.1.2 and 1.4.1.4] were measured as described by Polakis and Bartley (27). Succinate-cytochrome $c$ oxidoreductase (EC 1.3.99.1) was measured by a slight modification of the method of Mackler et al. (15). All measurements were carried out in cuvettes of 10-mm light path with a Beckman DB-G spectrophotometer at 35°C. The specific activities of the enzymes were calculated on the protein concentration (as bovine serum albumin equivalents) in cell-free extract and expressed as nanomoles of substrate transformed per milligram of protein per minute, i.e., as $10^4$ international units per milligram of protein.

Most of the biochemicals were purchased from C. F. Boehringer & Söhne (Mannheim, Germany), dl-Isocitrate (trisodium salt), glyoxylate (sodium salt), and oxidized cytochrome $c$ were obtained from Fluka (Buchs, Switzerland), and the acetyl-coenzyme A (CoA) was a product of Nutritional Biochemicals Corp. (Cleveland, Ohio).

**Results**

**Enzyme pattern during growth on glucose in batch culture.** The diauxic growth of *S. cerevisiae* is characterized by the fast fermentation of glucose and a high specific growth rate ($\mu$) of 0.42 in the first log phase, followed by the oxidation of the accumulated ethyl alcohol and a lower growth rate ($\mu = 0.14$) during the second exponential phase. The plotted values (see Fig. 1A) for the respiration quotient (RQ) of the growing cell population demonstrate the striking change from
fermentative (RQ = 12 to 16) to oxidative metabolism (RQ = 0.4 to 0.5) as soon as the glucose concentration approaches zero (21).

The values of the specific activities of the seven enzymes (Fig. 1B and C) are in agreement with the findings of other investigators (10, 27, 29). The activity of aldolase, an enzyme representative for glycolysis, was high during the fermentative growth phase and decreased by about two-thirds during the following oxidative phase. The enzymes responsible for oxidative metabolism and growth on ethyl alcohol were repressed during growth on glucose and derepressed during the second stage of growth. A factor of increase of about 10 was measured for succinate-cytochrome c oxidoreductase, one of 20 for malate dehydrogenase, and one of around 200 for isocitrate lyase and malate synthase. As already pointed out by Polakis and Bartley (27), the activities of the two glutamate dehydrogenases showed completely opposite courses. The NAD+-linked glutamate dehydrogenase, considered a degradative enzyme, showed a course similar to that of the tricarboxylic acid cycle and glyoxylate bypass enzymes, with a sixfold increase from the first growth stage to the second growth stage. On the other hand, the activity of NADP+-linked glutamate dehydrogenase, described as having a chiefly biosynthetic role (27), decreased during the slower growth on ethyl alcohol, to about one-eighth of the maximal value in the first log phase.

Although the general aspects of the changes in specific activity of the tricarboxylic acid-cycle and glyoxylate bypass enzymes and the NAD+-linked glutamate dehydrogenase (group I enzymes) on the one hand, and of NADP+-linked glutamate dehydrogenase and aldolase (group II) on the other hand were similar, a different time course of the changes was determined for each enzyme. Derepression of the various group I enzymes occurred at different times during the transition from glucose to ethyl alcohol metabolism. Increased (derepressed) formation of these five enzymes started in the following sequence: succinate-cytochrome c oxidoreductase, NAD+-linked glutamate dehydrogenase, malate synthase, isocitrate lyase, malate dehydrogenase. The synthesis of the enzymes was turned on at different metabolic states, which are characterized by RQ, specific growth rate, or specific oxygen uptake, Qco, and carbon dioxide release, Qco2 (21).

Growth and enzyme pattern in continuous culture as a function of the specific growth rate. The main and basic aspects of the aerobic growth of bakers' yeast in glucose medium in continuous culture are shown in Fig. 2A (8). A range of purely oxidative metabolism (RQ = 1.0 for a range of dilution rate from 0.0 to 0.19) and a range of gradu-

![Graph showing enzyme activities and growth patterns](image-url)

**FIG. 1.** Batch growth of Saccharomyces cerevisiae in 0.92% glucose medium NL 10 under aerobic conditions. (A) Time course of the growth curve (dry weight, X) of glucose (S) and ethyl alcohol concentration (A), and of the respiration quotient (RQ). (B) Course of the specific activities (SA; nanomoles of substrate transformed per milligram of protein per minute = 10-4 international units per mg of protein) of aldolase (Ald), succinate-cytochrome c oxidoreductase (SCR), isocitrate lyase (ICL), and malate synthase (MS) as function of growth stage. (C) Course of the specific activities (SA) of malate dehydrogenase (MDH), and NAD+ and NADP+-linked glutamate dehydrogenases (GluDH) as function of growth stage.
ally increasing fermentation (μ > 0.19) can be distinguished. Because of the onset of aerobic fermentation and subsequent production of ethyl alcohol, the dry weight (X) diminished with increasing growth rate, causing a nonlinear increase of the specific substrate uptake rate Qᵦ. The actual alcohol, fermentation occurs with the glucose uptake rate below 1.0 (μ = 0.30 and increased with the growth rate at values of μ > 0.30) (Fig. 2A). Ethyl alcohol concentration in the medium was below 10 μg/ml until μ = 0.18 and reached values of 3.0 mg/ml in the range of the dilution rate from 0.28 to 0.40. The specific oxygen uptake rate, Qₒₒ, increased linearly with the growth rate, reaching a maximal value of 140 ml of O₂ per g (dry weight) per hr at μ = 0.19, and it diminished with higher growth rates to 60 ml of O₂ per g (dry weight) per hr at μ = 0.42. We have discussed earlier this growth rate-dependent increase of fermentation and decrease of oxidation from a purely kinetic point of view (8). We assumed that relatively changed reaction velocities of the enzymes at metabolic branching points were the causes for different types of metabolism under various growth conditions.

However, the data on the specific activities, as presented in Fig. 2B and C, seem to indicate that the apparent repression of oxidation and the changeover of the metabolism to a fermentative stage were at least partially caused by changes in enzyme pattern. Again, the shape of the curves for specific activities was different for each enzyme. Aldolase was shown to exhibit gradual derepression with increasing growth rate. A fivefold increase in specific activity was determined. The five enzymes of oxidative or related pathways (group I) are subject to gradual repression with rising growth rate. The maxima of activity lay, for all five enzymes, between μ = 0.12 and 0.18, i.e., within the range of purely oxidative metabolism (RQ = 1.0). Succinate-cytochrome c oxidoreductase showed approximately a fivefold decrease in specific activity. The courses of specific activities of malate and NAD⁺-linked glutamate dehydrogenase were marked by a sharp decrease between μ = 0.18 and 0.25, with a slighter increase above this growth rate, resulting in a 10-fold repression. The glyoxylate bypass enzymes, isocitrate lyase and malate synthase, exhibited marked maxima at μ = 0.125 (about one-third of the maximal activities during growth on ethyl alcohol in batch experiment, see Fig. 1B). A sharp 50-fold decrease in specific activities between μ = 0.125 and μ = 0.25 was determined. The changes in specific activity of NAD⁺-linked glutamate dehydrogenase (Fig. 2C) were similar to the changes of aldolase, but showed a stronger derepression in the range from μ = 0.0 to 0.2 and a lower one above this growth rate.

A decrease in the specific activities for all of the group I enzymes was observed at growth rates below 0.10, with isocitrate lyase and malate synthase showing the greatest decrease. This may not be an effect of relatively higher enzyme inactivation at slow growth rates, but may represent a real repression—as for aldolase and NAD⁺-linked glutamate dehydrogenase—caused by a relatively higher synthesis of other enzyme proteins, e.g., alcohol dehydrogenase (EC 1.1.1.1) and isocitrate dehydrogenases (EC 1.1.1.41 and 1.1.1.42; von Meyenburg, unpublished data).

**DISCUSSION**

Enzyme pattern in populations of *S. cerevisiae* is correlated with the growth conditions. In the continuous culture experiments, the glucose concentration S (which is a function of the dilution rate D in balance with the substrate consumption) allows the cells to grow at a certain rate (equal to D for the steady states considered); in batch experiments, the type of substrate conditions the growth rates. The measured enzyme patterns correspond to the shift from fermentation to oxidation during batch growth as a function of time (21), and to the inverse shift with increasing dilution rates in continuous culture experiments (8). Metabolism and enzyme pattern do depend on the growth rate under glucose limitation. The actual glucose concentration S is very low for most of the D range (see Fig. 2A), and the values for the glucose concentration rate Qₛ had to be calculated before the relationship between the control enzyme pattern, the concentration of glucose, the catabolic rates (energy generation), and the anabolic rates (growth rate) could be considered. Qₛ, corresponding to the rate of the first reaction in glycolysis, is considered a measure of the rate of glycolysis and is informative with respect to the concentrations of intermediate metabolites.

A comparison of the enzyme pattern in batch and in continuous-culture experiments shows that the formation of the oxidative enzyme pattern during growth on ethyl alcohol is actually the same as during growth at a dilution rate of 0.14 [which is equal to μ (max) on ethyl alcohol during the second phase in batch] in spite of the different carbon source. For first log phase and growth at maximal growth rate in continuous culture (D = 0.42), the enzyme patterns necessarily coincide. This demonstrates the dependence of the control of enzyme pattern on the metabolic rate, which is possible with a certain type and concentration of substrate, rather than on the molecular nature of the substrate. The regulation of the enzyme forma-
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Fig. 2. Continuous culture of Saccharomyces cerevisiae in the synthetic medium NL 10 (*S*<sub>b</sub> = 9.2 mg of glucose/ml as limiting substrate) under aerobic conditions. (A) Steady-state values for dry weight (*X*), actual glucose concentration (*S*), and the respiration quotient (*RQ*) in function of the dilution rate (*D*), the specific growth rate (*µ*). In the range for *D* from 0.0 to 0.25, the glucose concentration is below 10 µg/ml. The course of the specific substrate uptake rate (*Q<sub>s</sub>*) is calculated from the values for *X*, *S*, *S<sub>b</sub>* and *D* (*Q<sub>s</sub>* = (*S<sub>b</sub> − *S*)/*X* × *D*) and expressed as milligrams of glucose consumed per milligram (dry weight) per hour. The ethyl alcohol curve is omitted for better clarity. In the range of *D* from 0.0 to 0.18, the ethyl alcohol concentration is a function of the rate of glucose consumption and subsequent equilibrium rates of metabolic routes, rather than of the glucose concentration as such (32). In similar continuous-culture experiments with *S. cerevisiae* by McMurrough and Rose (19), invertase (EC 3.2.1.26) was shown to exhibit a similar dependence on the growth rate under aerobic conditions and glucose limitation as malate dehydrogenase (maximal activity at *D* = 0.08 and a strong repression with increasing *D*). Under oxygen limitation (no aeration), the specific activity of invertase was four times lower at the same growth rate; for these conditions, a sixfold increase in specific glucose uptake rate can be calculated from the experimental data [under aerobic condition at *D* = 0.08, *Q<sub>a</sub>* was equal 0.22; under oxygen limitation at the same *D*, *Q<sub>a</sub>* = 1.33 mg of glucose metabolized per mg (dry weight) per hr].

In experiments with bacteria, the degree of repression of enzyme formation has been correlated to the relative rate of carbohydrate dissimilation (23), to the feeding rate of carbohydrates in linearly growing cultures (1), to the type of metabolism (4, 5), and to the specific growth rate (26) or to the mean doubling time (12). Repression of β-galactosidase in *Escherichia coli* increased in parallel with growth rate or substrate uptake rate under conditions of limitation by the carbon source. A similar dependence has been found for the glyoxylate acid cycle enzymes (12). Our results with *S. cerevisiae* are consistent with these findings and strongly suggest that enzyme pattern in yeast is actually regulated by catabolite or metabo-

light repression (16, 18).

We have no experimental data on the intracellular concentrations of intermediates at different growth rates. Yet, according to the rates of glucose consumption (*Q<sub>s</sub>*), of specific oxygen uptake (*Q<sub>o2</sub>*), and carbon dioxide production (*Q<sub>CO2</sub>* (7, 8), and to the specific growth rate (*µ*), as the sum of the anabolic rates, we can expect certain levels of the concentration of catabolites as adenosine phosphates, nicotinamide nucleotides, and glucose phosphates (22, 27, 28, 30),
and of metabolites [end products of the various pathways (18)], which were considered to be closely related to repression and derepression control.

One remark has to be made on the formation of the glyoxylate bypass enzymes in continuous culture. One would assume that the functioning of the glyoxylate bypass is not necessary, during growth on glucose, due to the probable preferential formation of \( C_4 \) skeletons by condensation of \( C_3 \) compounds and \( CO_2 \) (Wood-Werkman condensation). The two enzymes are possibly derepressed at low levels of phosphoenolpyruvate or a related compound at low glycolytic rates, as has been shown in \( E. coli \) (12), or at low levels of \( C_4 \) compounds because of low \( CO_2 \) concentration under the well-aerated conditions in the medium and limitation of the Wood-Werkman condensation (13). As already stated by others (32), induction by \( C_4 \) compounds can be excluded, because the concentration of ethyl alcohol is below 10 \( \mu g/ml \) in the critical range of dilution rate.

During the sequence of derepression of the group I enzymes in the batch experiment and the simultaneous repression of the group II enzymes during the shift from high growth rate on glucose to the necessary adaptation on a new substrate (ethyl alcohol) allowing only low growth rate, let us assume that the formation of each enzyme is subject to different thresholds of the controlling metabolic conditions. Corresponding to the metabolic, and thereby induced, enzymatic shift, a strong decrease in the percentage of budding cells in the population (from 95 to 25\%) has been detected (see Fig. 3). This has to be interpreted as an actual synchronization (20). The sequential derepression of the group I enzymes corresponds to the transition of most cells from a budding state to single cells. We assume that a relationship exists between the external conditions, the formation of the enzymes, and the state of the cells in the budding cycle. The appearance of the change of enzyme pattern after the fermentative-oxidative shift during the inter-log-phase and the second exponential growth phase elucidates the "unsteady state" type of growth (see Fig. 1). No constant levels appeared for any of the measured enzymes. This is not surprising, because the cells were growing only for 1.5 to 2.0 generations on ethyl alcohol. Oscillations of the specific activities (aldolase and NADP\(^+\)-linked glutamate dehydrogenase) or stepwise increases, due to oscillations in the rate of enzyme synthesis, can be recognized (Fig. 1B and C). A correlation between the oscillations in enzyme pattern, gas metabolism (see RQ in Fig. 1A and reference 21), and the population distribution (percentages of budding and single cells; see Fig. 3) can be assumed.

In continuous experiments with glucose limitation, the percentage of budding cells decreases with decreasing growth rate, and the percentage of single cells inversely augments (20). This indicates a change of the relative length of the budding phase and the single-cell phase in the individual cell cycle. At maximal growth rate, the duration of the budding phase equals the duration of the whole budding cycle (equal to the minimal generation time, which equals 1.5 hr). With increasing generation times, an elongating single-cell phase is inserted between scission of the daughter cell (bud) from the mother cell and the onset of another budding, whereas the length of budding period appears to remain approximately constant (Fig. 4). We suggest now, as we did on the basis of the batch results, the existence of an inherent correlation between the appearance of the morphological cell cycle and the determined enzyme pattern in asynchronous cultures under various degrees of glucose limitation. Sequential enzyme formation over the budding cycle was determined in yeast (9, 31), implicating sequential control of repression and derepression by cyclic changes of internal conditions. From these findings, we may conclude that different enzyme patterns under various growth conditions are the result of changed concentration sequences of catabolites or metabolites over the budding cycle, exhibiting control of repression or derepression at different thresholds for different enzymes. Studies on synchronized cultures of bakers’
yeast at different growth rates (under various degrees of glucose limitation) may elucidate the proposed dependence of the enzyme pattern on the relationship among individual cell cycle, sequential enzyme formation, and intracellular conditions.

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LITERATURE CITED


