Production of Maltase by Wild-Type and a Constitutive Mutant of Saccharomyces italicus

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Received 1 June 1981/Accepted 30 September 1981

The production of maltase, an inducible and repressible catabolic enzyme in Saccharomyces italicus, was studied and compared in batch, fed-batch, and continuous fermentations. Tight genetic controls on maltase synthesis limited the effect of environmental manipulations such as fed-batch or continuous culture in enhancement of maltase synthesis, and neither approach was able to improve the performance above the batch process for maltase production. S. italicus was mutated, and a constitutive producer of maltase was isolated. The mutant was detected by its ability to grow on sucrose, which is a noninducing substrate that is hydrolyzed by maltase; S. italicus does not possess invertase and will not normally grow on sucrose. Maltase production by this mutant was studied during growth on sucrose in batch and continuous cultures and marked improvement in enzyme productivity was observed. The specific activity of maltase produced by this mutant was more than twice that of the parent wild type: 2,210 and 1,370 U/g of cells for the mutant versus 890 and 510 U/g of cells for the wild type in batch and continuous cultures, respectively. Maltase specific productivity was increased from 74 to 288 U/g of cells per h by switching from batch growth of the wild type to continuous cultivation of the mutant.

Maltase, or α-glucosidase (EC 3.2.1.20), is an intracellular enzyme in Saccharomyces italicus. Its synthesis is induced by maltose and repressed by glucose. The enzyme cleaves the α-1,4-glycosidic linkages of maltose to release two glucose moieties. Thus, maltase provides a good model system for investigating the production of an enzyme under dual control by induction and catabolite repression. The objective of this project was to improve the production of maltase by S. italicus with both environmental and genetic manipulations.

Environmental manipulations affecting the growth rate, such as fed-batch or continuous culture, are often used to deregulate enzyme synthesis. Carbon-limited growth frequently stimulates the production of catabolite-repressed enzymes. Manipulations such as these would be expected to improve maltase production.

Another route to improvement involves genetic deregulation and selection of a constitutive mutant. One of the easiest selection procedures is one that will allow only the desired mutant to grow while other mutants and the wild type cannot survive. For example, growth on a noninducing substrate can be used to select for constitutive producers of catabolic enzymes. S. italicus does not normally utilize sucrose, presumably because it lacks invertase. Furthermore, sucrose will not induce maltase; however, maltase is able to hydrolyze sucrose and allow cell growth. Thus, a maltase constitutive mutant should grow on sucrose.

MATERIALS AND METHODS

Organism. The wild-type organism used in this study was S. italicus originally obtained from H. Halvorson at Brandeis University. This strain was deposited by H. Halvorson as ATCC 22185. A mutant strain, ATCC 20601, constitutive for maltase synthesis was derived from this parent.

Media and growth conditions. S. italicus was stored at 4°C on agar slants containing (grams per liter): yeast extract (Difco Laboratories, Detroit, Mich.), 10; peptone (Difco), 20; maltose, 20; and agar, 20. S. italicus was typically cultured in a chemically defined mineral salts medium containing: (NH4)2SO4, 8.9 g/liter; KH2PO4, 1.65 g/liter; MgSO4·7H2O, 1.3 g/liter; CaCl2, 0.1 g/liter; P-2000 antifoam, 0.2 ml; biotin, 2 μg/liter; pantothenate, 400 μg/liter; inositol, 2 ml; pyridoxine, 400 μg/liter; thiamine, 400 μg/liter; FeSO4·7H2O, 2.78 mg/liter; ZnSO4·7H2O, 2.88 mg/liter; CuSO4·5H2O, 1.6 mg/liter; Na2MoO4·2H2O, 2.42 mg/liter; CaCl2·6H2O, 2.38 mg/liter; and MnSO4·H2O, 1.69 mg/liter. Sterilization was effected by autoclaving at 121°C for 15 min; the carbon source and magnesium sulfate were autoclaved separately and added after cooling. The vitamins were filter-sterilized. The medium pH was adjusted to 5.5 with 2 N NaOH after autoclaving. Shake-flask cultures were inoculated with a single loop from the slant. After 18 h of growth, a
10% inoculum was used for the other flasks or the fermentor. The cultures were incubated at 30°C on a rotary shaker at 220 rpm. Generally, about 50 ml of growth medium was used in a 300-ml baffled flask to provide adequate oxygen transfer.

The fermentor was fabricated from a 4-liter Pyrex reaction kettle bottom (Fisher Scientific Co., Pittsburgh, Pa.) and used with a working volume of 2 liters. Temperature was controlled at 30°C. The aeration rate was approximately 1.0 (vol/vol) per min, and the agitation speed was adjusted so that the dissolved oxygen concentration never fell below 30% of air saturation. The pH was controlled at 5.5 ± 0.1 with the addition of 2 N NaOH as required.

For continuous culture, fresh medium was pumped into the fermentor at a constant rate, and culture broth was pumped out to maintain a constant volume. Samples were routinely taken from the outlet of the fermentor for measurements of culture turbidity, residual maltose, ethanol concentration, and maltase activity. Steady state was assumed when these values converged to constant readings.

**Analytical procedures.** The optical density of the culture was measured with a Klett-Summerson colorimeter with a red filter (Klett Manufacturing Co., New York). Samples were diluted as necessary to keep the reading between 10 and 150 Klett units. The factor of 185 Klett units per g of cells (dry weight) was used to convert between optical measurements and cell densities. Maltose was measured by the dinitrosalicylic acid reagent method of Miller (6). A phenolsulfuric acid assay (4) was used for determination of sucrose concentration. Ethanol was measured by using a Hewlett-Packard model 5830A gas chromatograph with an automatic injector and flame ionization detector. The column was packed with Chromosorb 101 (Johns-Manville, Denver, Colo.). Column conditions were: injector temperature, 250°C; column temperature, 135°C; detector temperature, 250°C; and carrier gas (helium) flow rate, 40 ml/min. The internal standard was n-propanol.

The cells were ultrasonically disrupted and centrifuged at 12,000 × g for 10 min at 4°C to remove cell debris, and the supernatant was used for enzyme assays. Maltase activity was determined by its hydrolysis of the (colorless) substrate p-nitrophenyl-α-D-glucoside (Sigma Chemical Co., St. Louis, Mo.) into glucose and p-nitrophenol which is detected by its absorbance at 400 nm. A total of 3 ml of the assay mix (1 mM p-nitrophenyl-α-D-glucoside in 0.07 M phosphate buffer [pH 6.8]) was placed in a tube in a 37°C water bath. After temperature equilibration, 20 μl of enzyme solution was added to initiate the reaction which proceeded until a yellow color was visible. At this point, the reaction was quenched with 3 ml of a 0.2 M NaHCO₃ solution. The absorbance was read at 400 nm by using distilled water as a blank. Maltase activity is expressed as:

\[
\text{maltase (U/ml)} = \frac{(11.075 \times \Delta \text{absorbance})}{\text{time of reaction}}
\]

where Δ absorbance is the difference between the absorbance of the sample and the blank, and 1 U is that amount of enzyme that will split 1 μmol of p-nitrophenyl-α-D-glucoside per min at 37°C.

**Mutagenesis.** *S. italicus* was mutagenized with ethyl methane sulfonate (Sigma Chemical Co., St. Louis, Mo.). Freshly grown cells were treated with a 3% ethyl methane sulfonate solution at pH 7 for 1 h. Ethyl methane sulfonate was then inactivated with 5% sodium thiosulfate. The cells were grown for several generations on a medium containing (grams per liter): yeast extract, 10; peptone, 20; and glucose, 20. The cells were then centrifuged, washed twice, plated on a selection medium containing (grams per liter): yeast nitrogen base (Difco B391), 6.7; sucrose, 20; and agar, 20. Surviving colonies were tested in liquid medium of the same composition without the agar. Cells were harvested after growth and sonicated, and the maltase activity was assayed.

**RESULTS**

The production of maltase by the wild-type *S. italicus* in a batch fermentation on maltose provides a base line against which future improvements may be judged. The mineral salts medium was used with an initial maltose concentration of 45 g/liter (Fig. 1). During the period between 0 and 13 h, the maximum specific growth rate was 0.45 h⁻¹. Ethanol accumulated until the maltose was exhausted at 13 h. The formation of ethanol results in a low cell yield on maltose: 0.15 g of cells per g of maltose. The maltase specific activity passes through a maximum value of 890 U/g of cells (dry weight) at the time that maltose is exhausted. Without maltose, maltase induction ceased, and the specific activity dropped during subsequent growth on ethanol. The spe-
specific activity decreased at a rate faster than can be ascribed solely to dilution by further growth. It appears that the enzyme was being inactivated or degraded while the cells were growing on ethanol. The inactivation was initially very rapid: 25% of the total activity was lost in the 1st h, followed by a slower first-order decay in total activity.

A fed-batch fermentation with maltose as the limiting nutrient was performed in an attempt to realize two objectives. First, the slow feeding of maltose may reduce or minimize catabolite repression and allow increased maltase expression. Second, a fed-batch operation can be used to eliminate ethanol production, thereby increasing the yield of cell mass and hopefully enzyme. The maltose feed rate was manually controlled to allow a growth rate of 0.24 h⁻¹. The feed rate was determined by the formula:

$$F = \frac{\mu X}{Y_{s/s}}$$

where $X$ is the cell density, $Y_{s/s}$ is the yield of cells on substrate, $\mu$ is the desired specific growth rate, and $F$ is the rate of sugar addition. The cell density was estimated hourly, and the feed rate was recalculated. The pump flow rate was constant; therefore, the pump was turned on and off for the length of time necessary to meet the feeding requirements. No more than 1 g of sugar was added at any one time, and the feeding pulses were equally spaced. The results of this fermentation are shown in Fig. 2; the actual growth rate was 0.22 h⁻¹. Maltose concentration was always below 1 g/liter, and only a small amount of ethanol was produced. A total of 45 g of maltose was fed, and the final cell density was 15 g/liter. The resulting cell yield was 0.33 g of cells per g of maltose, which was somewhat lower than expected but still much better than observed in batch fermentation. The maltase activity fluctuated during the fermentation but never rose much above 400 U/g of cells (dry weight), a value that was 50% of that obtained in batch culture. This result was opposite to what was expected. The experiment was repeated with controlled growth rates of 0.20 and 0.24 h⁻¹ with nearly identical results.

The study of maltase formation by *S. italicus* in a maltose-limited continuous culture had two objectives: (i) to examine maltase production during steady-state balanced growth as opposed to the quasi-steady-state unbalanced growth in the fed-batch culture and (ii) to use continuous culture to examine the effects of growth rate separated from those of maltose concentration on maltase production. The steady-state values of cell mass, ethanol, and residual maltose are shown in Fig. 3. As the dilution rate increased, the maltose and ethanol concentrations increased. The cell yield at a low dilution rate (0.1 h⁻¹) was less than the expected value of about 0.5 g of cells per g of substrate, probably because of the maintenance energy requirement. The cell yield at high dilution rates (0.2 to 0.4 h⁻¹) decreased as ethanol production increased. The effect of dilution rate on maltase activity is shown in Fig. 4. The range of maltase activity seen at steady state in continuous culture was similar to the ranges of values obtained during the unsteady-state fed-batch runs and was about 50% of the maximum specific activity seen in batch culture. The results for these three sets of experiments with the wild type are summarized in Table 1.
Since environmental manipulations were not effective in increasing the maltase activity, genetic deregulation of maltase synthesis was considered. After mutagenesis with ethyl methane sulfonate, surviving organisms were selected for growth on sucrose which could only occur if maltase was produced constitutively. A total of 75 surviving colonies were chosen at random for further study. One mutant (strain 1-4) was found to greatly overproduce maltase, and it was compared with the wild-type *S. italicus* grown on various carbon sources in shake flasks. The mineral salts medium was supplemented with 5 g of yeast extract per liter and 25 g of either sucrose, maltose, glycerol, fructose, or glucose per liter or 8.2 g of sodium acetate per liter. Cell samples were taken at midexponential growth and assayed for maltase (Table 2). The wild type only produced high levels of maltase when grown on maltose; only a basal level was present during growth on the other carbon sources. Strain 1-4 produced maltase during growth on all carbon sources tested. Rapidly utilized substrates, such as glucose or fructose, repressed maltase somewhat, but the activity was still very high compared with the wild-type basal level. The highest production occurred with sucrose as the carbon source.

This constitutive mutant was tested in a batch fermentation by using the mineral salts medium supplemented with 2 g of yeast extract per liter and by deleting the vitamins; the sucrose concentration was 45 g/liter (Fig. 5). The maximum specific growth rate was 0.32 h⁻¹, a lower value than that found for the wild type; this was possibly due to some adverse side effect of the mutagenesis or a transport limitation for sucrose. The maltase activity peaked just as the sucrose was depleted. However, catabolite inactivation in this mutant seems to be less of a problem than for the wild type; in the wild type, the total maltase activity rapidly decreased (only 25% of the maximum total activity remained after 10 h) after exhaustion of maltose. In this case, the total activity did not decrease after exhaustion of the sucrose; in fact, it increased somewhat. The drop in specific activity reflects a much lower rate of maltase synthesis during growth on ethanol and the dilution of existing maltase by subsequent growth. The most important result from this fermentation was that the maltase specific activity had more than doubled compared with the wild type: 2,200 compared with 890 U/g of cells. This high specific activity

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**TABLE 1. Maltase production by wild-type *S. italicus***

<table>
<thead>
<tr>
<th>Process</th>
<th>Sp act (U/g of cells)</th>
<th>Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(U/g of cells per h)</td>
</tr>
<tr>
<td>Batch</td>
<td>890</td>
<td>70</td>
</tr>
<tr>
<td>Fed batch</td>
<td>410</td>
<td>20</td>
</tr>
<tr>
<td>Continuous</td>
<td>510</td>
<td>100</td>
</tr>
</tbody>
</table>

**FIG. 4.** Maltase specific activity of wild-type *S. italicus* as a function of dilution rate.

**TABLE 2. Maltase production on various carbon sources**

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Maltase (U/g of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Mutant 1-4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>No growth</td>
</tr>
<tr>
<td>Maltose</td>
<td>870</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10</td>
</tr>
<tr>
<td>Acetate</td>
<td>10</td>
</tr>
<tr>
<td>Fructose</td>
<td>10</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
</tr>
</tbody>
</table>
resulted in a twofold increase in specific productivity.

Strain 1-4 was grown in continuous culture on the mineral salts medium with 30 g of sucrose per liter supplemented with vitamins (micrograms per liter): folic acid, 2; niacin, 400; and riboflavin, 200. The cell density, ethanol, and residual sucrose concentration profiles are presented in Fig. 6. The behavior of the mutant was similar to that of the wild type. Cell washout occurred at a lower dilution rate than for the wild type; this corresponds to the lower maximum specific growth rate observed in batch culture. The relationship between maltase activity and dilution rate is shown in Fig. 7. The characteristic bell-shaped curve was seen with a peak in activity between 0.1 and 0.2 h\(^{-1}\). The maximum volumetric productivity again occurred at the same point on the wild type (\(D = 0.21\) h\(^{-1}\)). The maltase specific activity, although much higher than that obtained from the wild type in continuous culture, was below the maximum value found during batch growth of the mutant. In fact, the extent to which this activity had been lowered was similar to that observed in the wild type. The results of the batch and continuous growth of the mutant are summarized and compared with the batch results for the wild type in Table 3.

**FIG. 6.** Results of a continuous culture of *S. italicus* strain 1-4 on sucrose.

**FIG. 7.** Maltase specific activity of *S. italicus* strain 1-4 as a function of dilution rate.

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### TABLE 3. Maltase production by the mutant and wild type

<table>
<thead>
<tr>
<th>Process</th>
<th>Sp act (U/g of cells)</th>
<th>Productivity (U/liter per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch (wild type)</td>
<td>890</td>
<td>70</td>
</tr>
<tr>
<td>Batch (strain 1-4)</td>
<td>2,200</td>
<td>170</td>
</tr>
<tr>
<td>Continuous (strain 1-4)</td>
<td>1,400</td>
<td>290</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Maltase production by wild-type *S. italicus* is highly regulated, making its overproduction difficult in response to various environmental manipulations. Maltase specific activity peaks during batch growth just as maltose is exhausted from the medium and the specific growth rate decreases. During the early exponential growth phase, when growth was most rapid, the enzyme level is low. This is most likely a result of catabolite repression which adversely affects maltase expression; catabolite repression is known to exert its strongest effects at high growth rates (2).

The use of a fed-batch strategy to maintain a low maltose concentration and circumvent catabolite repression was not successful. Even though the growth rate was controlled at a level below that expected to cause catabolite repression (0.20 to 0.24 h\(^{-1}\)), maltase activity was only 50% of the maximum value found in batch culture. One possible explanation involves the operating method used for the fed batch. Maltose was added periodically by pulse-addition of a sugar solution by a preset schedule, rather than by using a continuous feed. It is possible that unbalanced growth resulting from discontinuous feeding caused a decrease in maltase synthesis. Welles and Blanch (10) found that pulse-feeding glucose to *S. cerevisiae* caused a drop in cell yield and an increase in ethanol production. However, results obtained at steady state in continuous culture suggest that the low maltase activity observed in fed-batch culture probably was not because of periodic feeding or other transients occurring during the fed batch. The specific activity was again approximately 50% of the maximum value found in batch culture. A more likely hypothesis is that the low residual maltose concentration present in both the fed-batch and continuous cultures resulted in only a partial induction of maltase. This argument is
supported by the observation that by increasing the dilution rate from 0.1 to 0.2 h⁻¹ both the residual maltose concentration and the maltase specific activity increased. An alternate explanation is that maltase catalyzes the growth-limiting reaction, and the increased activity is required to achieve an increasing growth rate until catabolite repression limits the activity at high dilution rates. However, a calculation shows that the rate of maltose utilization theoretically permitted by the maltase present is 5- to 10-fold greater than that needed for growth. If the maltose concentration is assumed to be equal to the value measured in the broth, 8.7 x 10⁻³ M, then the enzyme activity in vivo was 20% of the maximum value measured (K_m for maltose = 3.5 x 10⁻² M). Since active transport of maltose undoubtly results in a higher internal concentration, this hypothesis is less likely.

Maltase synthesis appeared to be under dual control (3) by both induction and catabolite repression. Induction by maltose causes the specific activity initially to increase with the dilution rate, and at growth rates above 0.2 h⁻¹ catabolite repression causes a concomitant decrease in enzyme activity and permits increased ethanol formation. The net effect of both controls is that the specific activity peaks at some intermediate value of growth rate. Invertase in both S. cerevisiae and S. carlsbergensis behaves in a similar manner (5, 8, 9); the specific activity and volumetric productivity pass through a maximum value, reflecting the dual control of induction and catabolite repression. The dilution rate at which this occurs is the same as that observed here for maltase: approximately 0.2 h⁻¹.

The constitutive mutant selected for by growth on sucrose produced more maltase than did the wild type and no longer had an absolute requirement for maltose as the inducer. However, maltase synthesis still appeared to be regulated by the dual control of induction and catabolite repression. Catabolite repression apparently caused a decrease in activity at high dilution rates and during early exponential growth in batch culture. The specific activity in continuous culture had the same bell-shaped profile as that of the wild type, indicating that induction may still be important even though constitutivity has been demonstrated. Maltase synthesis is under positive control (1, 7); maltose must complex with a specific regulatory protein and bind to a segment of the operator for transcription to occur. Thus, the most likely result of mutagenesis in strain 1-4 was alteration of the conformation of this protein so that it could bind to the DNA and promote transcription without the need for maltose. The various maltase activities produced on different carbon sources may be a measure of their effect on the synthesis of this regulatory protein, since it is produced in a near-limiting concentration, activity by the wild type, or both. Alternatively, the modified regulator may have been able to interact with certain sugars to form a more potent inducing complex. Likewise, the initial increase in specific activity with the dilution rate could have been because of growth rate effects on the regulatory protein or the ability of sucrose to act as an inducer. The exact reason for this behavior cannot be determined until more information on the nature of the mutation has been elucidated.

LITERATURE CITED