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Invertase Production Is Related to the Nitrogen Source in *Hansenula anomala*

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Abstract

Differences in invertase accumulation of *Hansenula anomala* cultivated in ammonium and nitrate are reported. Media supplemented with sucrose and ammonium sulfate as the sole carbon and nitrogen source, respectively, show strong media acidification (pH 3.0 or lower), and vigorous cell growth. Invertase activity was not detected under such conditions. A cell-free imitation experiment suggests that, under such low pH, extensive chemical breakdown of sucrose (>22%) occurs. Thus, *H. anomala* is able to grow under strong acidic conditions that permit sucrose metabolism by the uptake of monosaccharides generated from chemical hydrolysis. In addition, invertase activity is not present in cells grown in nitrate as nitrogen source at pH 5.0, but at pH 7.0 activity is detected. If ammonium is supplied instead of nitrate, cells grown at pH 5.0 show invertase activity and at pH 7.0 high levels of activity are detected. These results indicate a specific physiological response of the sucrose metabolism to the presence of alternate nitrogen source.

Index Entries: *Hansenula anomala*; invertase; nitrogen source; sucrose metabolism.

Introduction

Invertase and the microorganisms that produce it are used in many ways in biotechnology to process sucrose. The assimilation of sucrose by *Saccharomyces cerevisiae* and many other yeasts involves, in the first step, the extracellular breakdown of the sucrose molecule to glucose and fructose. In the second step, the monosaccharides are transported into the cell to feed the central glycolytic pathway *(1)*. Invertase is the major enzyme involved in the breakdown of sucrose in many yeast. In *S. cerevisiae*, the SUC 2 gene encodes two forms of invertase, a secreted and an intracellular enzyme *(2)*. The secreted enzyme is heavily glycosylated, derepressible, and plays the

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major role in sucrose metabolism *(3)*. Extracellular invertase is controlled by carbon catabolite repression at the transcriptional level *(4,5)*.

Hansenula anomala, unlike many other yeasts, assimilates nitrates as a sole nitrogen source *(6,7)*. In most microorganisms, nitrate metabolism proceeds via the inducible nitrate reductase pathway *(8–10)*. Nitrate reductase is induced by the presence of nitrate and repressed by the addition of ammonium *(11)*.

In initial experiments we noticed that *H. anomala* grows well on sucrose independently of the nitrogen source, ammonium, or nitrate. In ammonium cultures we were, however, unable to detect invertase activity. In this article, we show that under normal conditions, the uptake of ammonium decreases the extracellular pH to a level in which sucrose is inverted chemically by the acidic conditions of the media. In conditions in which the media pH is buffered, higher invertase production is shown, indicating a specific physiological response related to the nitrogen source.

Materials and Methods

Yeast Strain

H. anomala IZ 1420 (Instituto Zymotecnico-ESALQ/USP, Piracicaba, Brazil) was used throughout this study. Stock cultures were maintained in YED (10 g/L yeast extract, 10 g/L glucose, 15 g/L agar) slants and stored at 5°C.

Buffers

Acetate, phosphate and Tris/HCl buffers 100 m*M* were used to cover the pH ranges from 3.5–6.0, 5.5–7.7, and 7.0–8.0, respectively.

Growth Conditions

Inoculum was prepared by growing a loopful of cells from YED slants in a 25-mL glass test tube containing 5 mL of YED broth, which was shaken at 150 rpm for 14 h at 30°C. The culture was then kept overnight in 50 mL of Wickerham medium supplemented with 10 g/L glycerol *(12)*. Inoculum was then seeded at an initial density of 10^7 cells/mL in a 300 mL Erlenmeyer flask containing 50 mL of Wickerham media supplemented with 1 g/L sucrose and ammonium sulfate or potassium nitrate to a final concentration of 50 m*M*. Aliquots were taken from time to time to determine growth parameters and invertase activity. Kinetic data were obtained according to Borzani *(13)*.

Measurement of Invertase Activity in Whole Cells

Invertase activity was carried out by adding 10–50 µL of acetate or phosphate buffer-washed cells into a mixture containing 20 m*M* sucrose dissolved in 100 m*M*, acetate buffer, pH 5.0, or phosphate buffer, pH 7.0, to a final volume of 1 mL. The reaction was incubated at 30°C for 60 min and

heat-inactivated. Production of glucose was determined using the glucose-oxidase method *(14)*. One enzyme unit (U) was defined as the amount of enzyme necessary to release 1 μmol of glucose produced/min/mL of the reaction mixture. The uptake rates of glucose by cells added to the reaction mixture was determined by comparing the activity of reactions done in the absence and presence of antimycin A 0.5 μ*M*, a respiratory inhibitor *(15)*.

Chemical Inversion of Sucrose in a Cell-Free System

Imitation of physiological conditions in a cell-free system was performed in Wickerham medium supplemented with sucrose and ammonium sulfate. The pH was adjusted with 0.1 *N* sulfuric acid or acetic acid. Filter-sterilized medium was incubated at 30°C at standard conditions in the absence of cells and the amount of released glucose determined by glucose-oxidase method *(14)*.

Other Methods

Growth was determined measuring the cell density spectrophotometrically at 600 nm or by total protein determination. Total protein was determined as described by Bradford *(16)*. Glucose was determined by the glucose-oxidase method *(14)*. Sucrose was determined by measuring the net formation of glucose in chemically inverted samples. To 1 mL of media, 1 mL of 2 *N* HCl was added and heated at 68°C for 10 min and glucose was determined on neutralized samples. Complete inversion under these conditions was analyzed by high-performance liquid chromatography (HPLC). Ammonium concentration was determined by the micro-Kjeldhl method *(17)*. Raffinose and corresponding hydrolytic products were analyzed by HPLC, using a Bio-Rad Aminex HPX 87H column and 0.1 *N* sulfuric acid as the eluant.

Results

The invertase activity determined in this study refers to the activity present at the outer surface of living *H. anomala* cells. We believe that the activity detected under our experimental conditions is related to an β-D-fructofuranoside fructohydrolase. Quantitative enzymatic activity comparisons, using melibiose, raffinose, and maltose as substrates and determination of the hydrolysis products of reactions containing raffinose, indicate that the predominant activity is invertase (Medeiros and Prade, unpublished results). No significant differences (less than 5%) was observed in the amount of glucose detected in the enzymatic reaction mix by adding 0.5 μ*M* of Antimicin A.

In initial experiments, we noticed that *H. anomala* grows at similar rates in sucrose-containing media supplemented with ammonium or nitrate. Table 1 shows typical growth kinetic data related to invertase activity. In both nitrogen sources, sucrose was extensively utilized and cell mass increased substantially. Surprisingly, no invertase activity was present in

Table 1
H. anomala Utilizes Sucrose to Grow Without Detectable Invertase Activity When Ammonium Sulfate Is Used as the Sole Nitrogen Source

	Growth parameters			
Nitrogen source	μ[a]	Yx/s[b]	Sucrose consumption (%)[c]	Invertase activity (U. mg/prot)
Ammonium	0.19	0.47	38.34	0.006
Nitrate	0.28	0.41	67.19	0.28

[a]μ, specific growth rate at the log fase.
[b]Yx/s, yield of cell mass in relation to the consumption of sucrose ($g.g^{-1}$).
[c]100%, 1 g/L sucrose.

cell suspensions that had been cultured in the presence of ammonium. After 14 h of growth, however, utilization of 38.34% of the initial sucrose content occurred to yield 0.47 g of cells for each g of sucrose assimilated. To ensure that under such conditions sucrose was assimilated and extensively metabolized, we tested the metabolic adaptation to alcoholic fermentation. After 72 h, under semianaerobic conditions, in the presence of sucrose and ammonium, the system produced 0.42 g of ethanol and 0.05 g of cells for each g of sucrose utilized, indicating a prompt switch to fermentation and vigorous sucrose metabolism

To determine the direct relationship among growth, invertase activity, and the sucrose content, cells were incubated in ammonium- or nitrate-containing media with increasing amounts of sucrose. Figure 1 shows growth and invertase activities after 14 h of incubation. In both nitrogen sources, growth was similar but invertase was not detected in ammonium-containing media. Invertase activity accumulates with the increase of sucrose in nitrate-containing media. Figure 2A shows the final pH and the consumption of ammonium ions related to the presence of increasing amounts of sucrose. The final pH decreases sharply with the uptake of ammonium ions and is dependent on the sucrose concentration up to 20 m*M*. At higher sucrose contents, the pH remains constant. Under such conditions, growth is limited by the presence of sucrose and related to the acidification of the media by the uptake of ammonium ions. In Fig. 2B, the acidic conditions were imitated artificially by adding sulfuric acid to a cell-free, physiologically equivalent system. The chemical inversion of sucrose was determined and extensive hydrolysis (>20%) was observed at pH values lower than 3, suggesting that under the conditions studied in Table 1 and Fig. 1, sucrose is hydrolyzed chemically. Cultures that received potassium nitrate did not alter the pH throughout the incubation period (data not shown).

In Fig. 3, the comparison of invertase activity produced in cultures grown in the presence of nitrate or ammonium under different pH conditions is shown. Values express the amount of enzyme produced in different

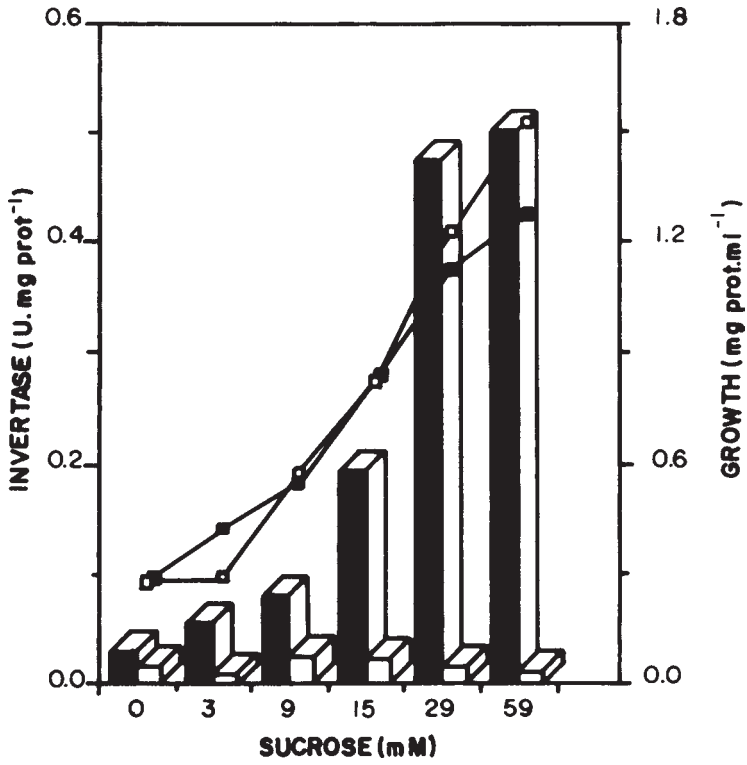

Fig. 1. Effect of sucrose concentration on invertase activity in *Hansenula anomala* grown in the presence of nitrate or ammonium salts. Invertase activity (bars) and growth (lines) was determined from cells grown in Wickerham media, supplemented with sucrose as indicates, 50 m*M* ammonium sulfate (open symbols or white bars) or potassium nitrate (closed symbols or black bars).

pH's, whereas activity was assayed at pH 7 and related to the total cellular protein content. For comparative purposes, values may not represent equal amounts of enzyme, if kinetic differences exist between ammonium and nitrate prepared cells (e.g., presence of more than one activity). Cells grown at pH 5, produce invertase in the presence of ammonium, but do not accumulate the enzyme in nitrate-containing media. At pH 7, nitrate-grown cells produce invertase, but in ammonium, cells accumulate it twice as much. In Fig. 4, we compare invertase activities assayed in different pH conditions of ammonium- and nitrate-grown cells. Cells were grown in the presence of nitrate or ammonium, in pH 5 or pH 7 buffered media containing sucrose. Invertase activity was determined in the pH range shown in Fig. 4. Values report changes in invertase-specific activity related to kinetic alterations induced by pH, while a constant amount of cells was used. Cells grown at pH 5 in the presence of nitrate are not active for invertase in any of the tested pHs, but cells grown in ammonium are, as expected (Fig. 3). Although activity was high for cells grown at pH 7 in both nitrogen sources, pH-dependent activity profiles were different. Cells

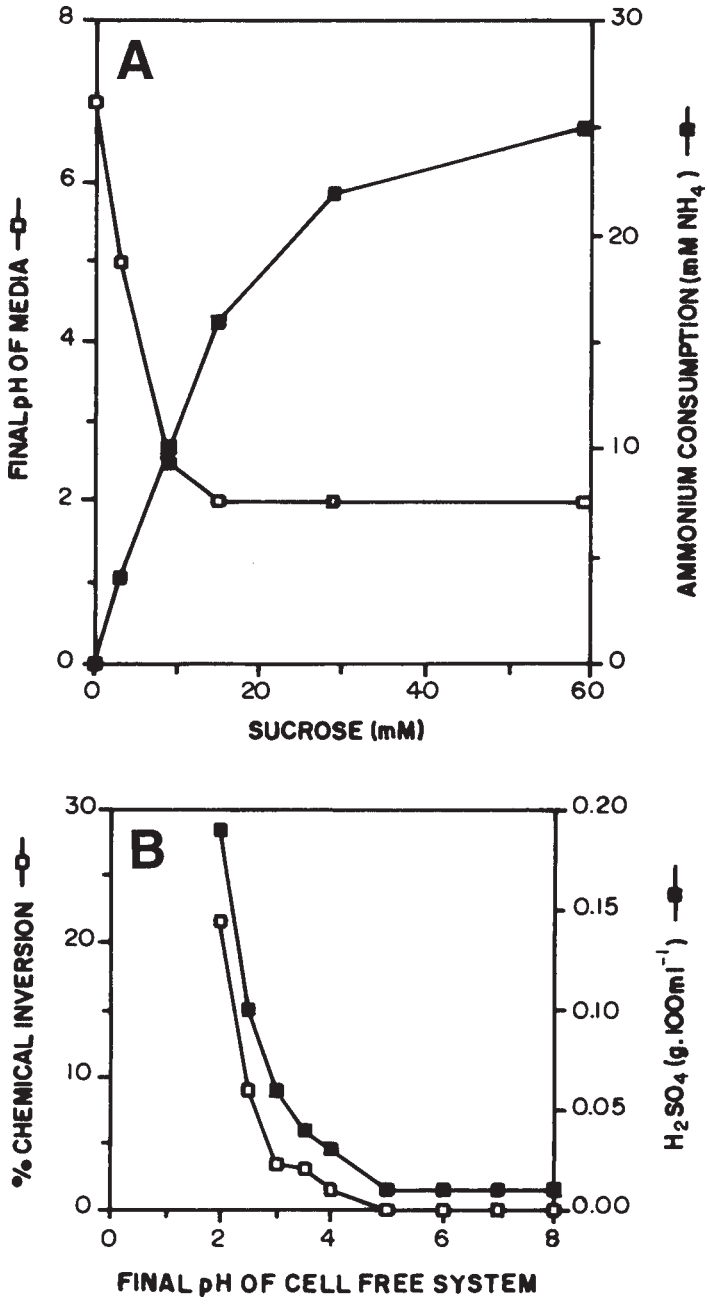

Fig. 2. Chemical breakdown of sucrose occurs in cultures grown in the presence of ammonium. **(A)** shows the consumption of ammonium ions (closed symbols) and the reduction of pH (open symbols) related to the initial concentration of sucrose, present in cultures grown with 50 m*M* ammonium sulfate. **(B)** An in vitro system is shown to demonstrate that chemical inversion of sucrose can occur under the conditions of (A). The pH of media containing sucrose 10 g/L and 50 m*M* ammonium sulfate was adjusted as indicated in the figure, biological cultivation conditions imitated and the amount of release glucose determined.

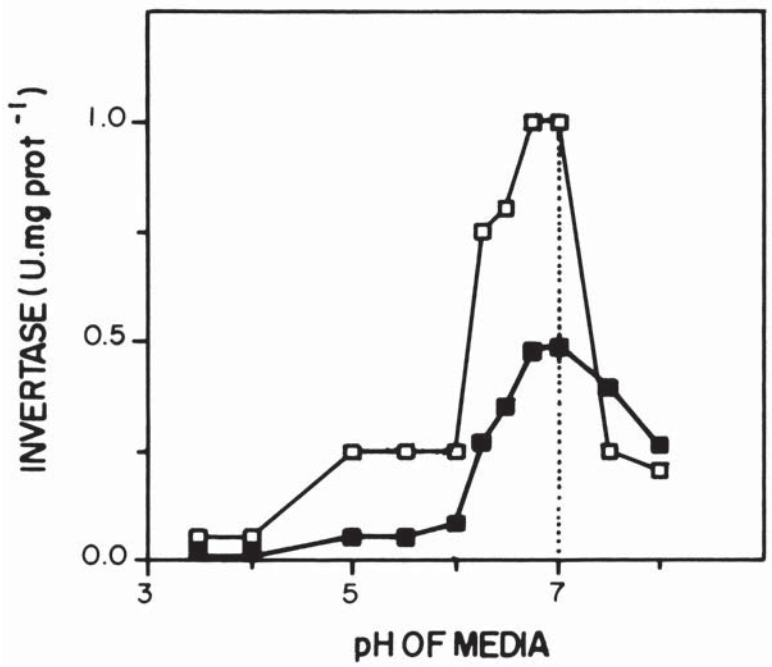

Fig. 3. Comparison of invertase-accumulation profiles of cells grown in the presence of nitrate or ammonium under different pH conditions. Invertase activities, determined from cells grown in media buffered at the indicated pHs, containing sucrose, ammonium sulfate (open symbols) or potassium nitrate (closed symbols) are shown. The pH of all enzymatic reactions was 7.0.

grown in ammonium have an extra peak of activity when compared with cells grown in nitrate.

Discussion

The data presented in this article permit two conclusion to be drawn concerning the sucrose metabolism in *H. anomala*. First, the evidence suggests that under acidic conditions *H. anomala* grows well by utilizing monosaccharides generated from chemical hydrolysis of sucrose, and second, the form of inorganic nitrogen available, alters biosynthetic or kinetic properties of the sucrose hydrolytic system. The absence of invertase activity when nitrogen is supplied in the form of ammonium ions could be partially explained by assuming an unstable enzyme that is inactivated and/or inhibited during cultivation and/or sample processing. Figure 2A,B, however, suggest that under the conditions of study, sucrose is hydrolysed chemically by the strong acidic conditions generated by the uptake of ammonium ions required for growth. Figure 3 in which the media was buffered also shows that little or no invertase is synthesized at pH values under 4 and Fig. 4 shows that activity can be detected in reactions in which the pH is lowered to 3.5. Therefore it is likely that acidic environ-

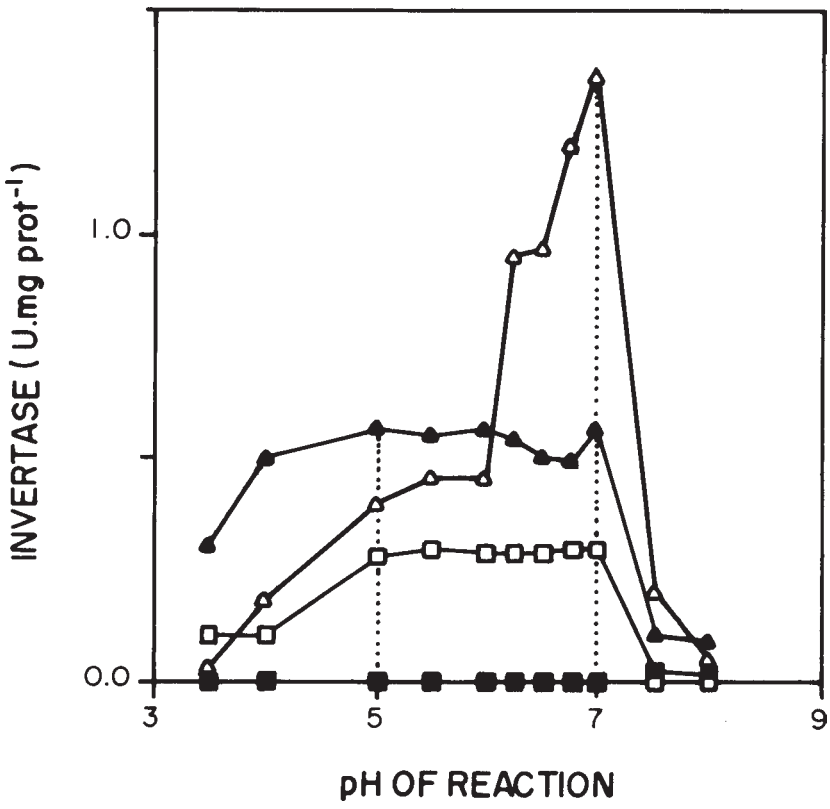

Fig. 4. Comparison of the variation of invertase activity with pH of cells grown in nitrate and ammonium. Cells were grown with the addition of nitrate (closed symbols) or ammonium (open symbols), in media buffered at pH 5.0 (squares) or 7.0 (triangles). For each sample, enzyme activities were determined in reactions buffered at the indicated pHs.

ments are physiologically adequate for this organism and sucrose is metabolized in the absence of invertase by chemical breakdown.

Invertase-production profile comparisons (Fig. 3) indicate that in ammonium, accumulation of enzyme is composed of a two-phase curve. The nitrate-accumulation pattern is composed of only one phase, that overlaps with the second phase from ammonium-grown cultures. Thus, if the reported values express the amount of cell-wall accumulated enzyme, one possible interpretation of these differences could be that invertase is controlled by the form of nitrogen delivery. The data could also reflect kinetic differences. In the second case, it is likely that more than one enzyme is responsible for the invertase activity in this microorganism. Specific induced changes in kinetic properties of the invertase content in nitrate- and ammonium-grown cells could lead to the resolution of multiple enzymatic forms. In Fig. 4, kinetic modifications were induced by altering the pH of the reaction mixture. Cells grown at pH 5 and pH 7 buffered media containing potassium nitrate or ammonium sulfate were used. Figure 4

confirms the prediction that the invertase fraction of nitrate- and ammonium-grown cells, basically differ in their kinetic properties and therefore our data may indicate the presence of more than one enzyme that hydrolyzes sucrose. Nevertheless, our data suggest a specific physiological response of the sucrose metabolism to the presence of alternate nitrogen sources. Thus, this research illustrates the fact that *H. anomala* differs in many ways by which other yeasts respond to environmental cues and metabolize sucrose.

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