0273-2289/96/5758—0407$6.50

Hexokinase Production from *S. cerevisiae*

Culture Conditions

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ABSTRACT

The effects of pH (4.0, 4.5, or 5.0), temperature (*T*) (30, 35, or 40°C) and dissolved oxygen (DO) (0.2, 2.0, 4.0, or 6.0 mg O_2/L) on hexokinase and invertase formation by yeast were studied. The highest enzyme activities were attained at pH 4.0, DO = 4.0 mg O_2/L, and *T* = 35 or 40°C.

Index Entries: Hexokinase; invertase; *S. cerevisiae*; baker's yeast.

INTRODUCTION

Hexokinase (EC 2.7.1.1), a key enzyme in carbohydrate metabolism, catalyzes the conversion of glucose into glucose-6-phosphate (G-6-P). The G-6-P, in turn, is an intermediate for several metabolic pathways, including glycolysis. Hexokinase (HK) has an important role in the control of the catabolic repression *(1,2)*, as well as on the glucose uptake mechanism through the cytoplasmic membrane *(3,4)*. Furthermore, in yeasts, the HK is found to be predominantly solubilized in the cytoplasm *(5)*.

In addition to its importance in biochemical studies, HK is used in analytical methods for measurement of glucose, fructose, mannose, ATP, and creatin-kinase activity *(6)*. Furthermore, since HK allows for the measurement of glucose concentrations in the presence of fructose, it constitutes an important tool in the wine and fruit juice industries for the detection of illegal sugar additions in the final products *(7)*.

This article deals with the effect of pH, temperature, and dissolved oxygen on the HK production by *Saccharomyces cerevisiae*. In addition, the effect of these culture conditions on invertase activity of intact cells is also reported.

MATERIALS AND METHODS

Inoculum Preparation

S. cerevisiae (isolated from pressed yeast cake) was maintained on slant tubes containing 23.0 g/L nutrient-agar (Difco, Detroit, MI) and 1.0 g/L glucose. The cells were transferred to test tubes containing 2.5 mL of growth medium

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(GM) (10.0 g/L glucose, 5.0 g/L peptone, and 3.0 g/L yeast extract, pH 4.5) and incubated for 48 h at 33°C. Following this procedure, one tube was used to inoculate 50 mL culture medium (CM) in a 250-mL Erlenmeyer flask, followed by incubation at 30°C for 22 h in a rotary shaker (100 rpm) (Super-Hohm, Piracicaba, SP). The composition of *CM* was: 3.0 g/L yeast extract, 5.0 g/L peptone, 2.0 g/L glucose, 15.0 g/L sucrose, 2.4 g/L $Na_2HPO_4 \cdot 12H_2O$, 0.075 g/L $MgSO_4 \cdot 7H_2O$, and 5.1 g/L $(NH_4)_2SO_4$. All media were sterilized at 120°C for 20 min.

Batch Fermentation

A volume of 0.45 L of inoculum (0.70 g dry cell/L) was poured into a 5-L NBS-MF 200 bench fermenter (coupled with NBS dissolved oxygen controller, DO-81) containing 2.55 L of CM. The culture was then carried out at a temperature of 30, 35, or 40°C; impeller speed = 400 rpm, 0.1 mg/L dimethylpolysiloxane (addition dropwise when needed), pH 4.0, 4.5, or 5.0, and dissolved oxygen (DO) 0.2, 2.0, 4.0, or 6.0 mg O_2/L. Every hour, an aliquot of 20.0 mL of the fermenting medium was taken for analysis.

In all tests, the pH of the medium during fermentation was maintained at the desired value by automatic addition of 1*M* NaOH and 0.5*M* H_2SO_4.

Measurement of Glucose and Cell Concentrations

Five milliliters of fermenting medium were filtered through a Millipore membrane (pore diameter = 0.45 μ). The cell concentration, expressed as g dry cell/L, was measured by drying the cell cake (105°C for 2 h). The glucose concentration in the filtrate was measured by an enzymatic method using the system glucose oxidase (GOD)/peroxidase (POD) according to Bergmeyer *(6)*.

Measurement of Enzymes Activities

Invertase

The invertase activity determinations (always in duplicate) were carried out at 37°C in a mixture of 1.5 mL 0.010*M* acetate-acetic acid buffer (pH 4.6), 2.5 mL 0.3*M* sucrose solution, and 0.5 mL cell suspension. After 3 min, the hydrolysis was stopped by adding 1.0 mL of the Somogyi reagent *(8)* quickly followed by immersion in a boiling water bath for 10 min. The reducing sugar concentration (RS) was then measured as described previously *(9)*.

One invertase unit (U) was defined as the amount of enzyme catalyzing the formation of 1 g of RS/min at the assay conditions. Specific invertase activity (IA) was expressed as U/g dry cell.

The cell suspension was prepared as follows: 5 mL of the fermenting medium was centrifuged (3000*g*/15 min); the sediment was washed with distilled water, centrifuged (3000*g*/15 min), and suspended in distilled water in order to obtain a known suspension volume.

Hexokinase

A volume of 10 mL of fermenting medium was centrifuged (5000*g*/10 min), the sediment was washed with 3.0 mL of TRIS-HCI buffer (50 m*M*, pH 7.5), centrifuged (5000*g*/10 min), and resuspended in the same buffer (3.0 mL) containing: 2 m*M* $MgCl_2$, 0.2 m*M* EDTA, 2 m*M* aminocaproic acid, 2 m*M* DTT, and 1 m*M* PMSF. The cells were disrupted by submission to a vortex (PHOENIX AT56) in the pres-

Table 1
Variation of the Specific HK Activity, Specific IA, and tg for Batch Cultures of *S. cerevisiae* Carried Out at Different DO Concentrations, T = 35°C and pH = 4.5

		HK, U/mg dry cell			IA, U/g dry cell		
O_2, mg/L	tg, h	0 h	2.5 h	5.0 h	0 h	2.5 h	5.0 h
0.2	2.31	0.062	0.076	0.238	0.504	0.244	0.608
2.0	1.47	0.072	0.520	0.540	0.576	0.200	1.22
4.0	1.39	0.075	0.360	0.480	0.610	0.530	2.70
6.0	1.58	0.080	0.230	0.280	0.553	0.152	1.02

ence of 3.0-mL glass beads (0.5 mm diameter). Cell debris and glass beads were removed by centrifugation (5000*g*/10 min), and the supernatant was collected.

The HK activity was measured in the supernatant by spectrophotometric determination at 30°C of reduced NADP according to the method described by Bergmeyer *(6)*.

One HK unit (U) was defined as the amount of enzyme catalyzing the reduction of 1 μmol of NADP/min at the assay conditions. Specific HK activity was expressed as U/mg dry cell. The coefficient of variation (the ratio between the standard deviation and the HK activity mean value) was about ±5%.

RESULTS AND DISCUSSION

As can be seen from Table 1, dissolved oxygen (DO) clearly affects the specific HK activity of *S. cerevisiae* in the DO interval studied (from 0.2 to 6.0 mg O_2/L). HK of about 0.54 and 0.48 U/mg dry cell were attained at DO 2.0 and 4.0 mg O_2/L, respectively. Furthermore, a diminution of approx 50% of HK was observed at the upper and lower DO limits. This can be explained by taking into account that the pair HK/O_2 can be related to the glucose uptake and conversion into energy by yeast. Probably, the glucose/energy conversion should be optimized at 2.0 mg O_2/L ≤ DO ≤ 4.0 mg O_2/L by the fact that cellular growth, which was considered in terms of decreased generation time (tg), was markedly stimulated in that DO interval. The tg was calculated as proposed by Borzani *(10)*.

Since sucrose (a nonfermentable sugar) was the main carbon source present in the culture medium, the availability of glucose for the yeast depends on sucrose hydrolysis by invertase, an enzyme located at the yeast cell wall *(11)*. The invertase (EC 3.2.1.26) represents commercial interest owing to its generalized use for inverted syrup production and in sensors for continuous sucrose determination *(12,13)*. Thus, the invertase production was also considered.

From Table 1 it can be seen that the highest specific invertase activity (IA) (2.70 U/g dry cell) and the lowest tg (1.39 h) occurred at DO = 4.0 mg O_2/L. From the same table, a decreasing of IA is also observed around 55% at DO equal to 2.0 or 6.0 mg O_2/L and an average variation of tg of about 9%, as DO ranged from 2.0 to 6.0 mg O_2/L.

Table 2 indicates that at 35°C, the highest IA (2.70 U/g dry cell) was attained at pH 4.5. However, at the same temperature, the specific invertase activity diminished around 50 and 82% at pH 4.0 and 5.0, respectively. At first, the effect of pH on

Table 2
Variation of the Specific HK Activity, Specific IA, and tg for Batch Cultures of *S. cerevisiae* Carried Out at DO = 4.0 mg O_2/L and Different Temperature and pH

		HK, U/mg dry cell			IA, U/g dry cell			
pH	T, °C	0 h	2.5 h	5.0 h	0 h	2.5 h	5.0 h	tg, h
4.0	35	0.289	0.619	0.754	0.482	0.789	1.36	1.58
	40	0.370	0.560	0.780	0.488	0.207	0.703	1.82
4.5	30	0.260	0.180	0.410	0.336	0.160	0.982	1.73
	35	0.075	0.360	0.480	0.610	0.530	2.70	1.39
	40	0.120	0.300	0.500	0.476	0.380	0.658	2.04
5.0	35	0.149	0.185	0.549	0.325	0.220	0.486	2.10

IA could occur either intracellularly or extracellularly. As the pH was varied from 4.0 to 5.0, a change in the intracellular pH would be unlikely because *S. cerevisiae* has an effective internal buffering capability *(14)*. It would be acceptable therefore to presume that the pH had affected the enzyme activity extracellularly. Thereby, the invertase could have its tertiary and/or quaternary structures modified during budding leading to an inadequate insertion into the cell wall *(15)*.

Figure 1 shows that the reducing sugar concentration (RS) in the medium during yeast growth depends on the DO. Therefore, at the fermenting time between 0 and 2 h, the lowest increase in IA occurred in the test carried out at DO = 4.0 mg O_2/L, at which the average RS was about 6.0 g/L. It was also during this time when the highest final IA was attained (Table 1). Such behavior of IA may be the result of a glucose repression/derepression mechanism *(16)*. On the other hand, the HK activity was not affected by RS concentration in the culture medium (Tables 1 and 2, Fig. 1).

It is interesting to note from Fig. 1 that the RS in the medium increases up to t = 2 h, but decreases afterward. The accumulation of RS could be the result of sucrose hydrolysis by the invertase present in the inoculum cells and to the low cell concentration at this phase of the growth.

Table 2 indicates that at 35 and 40°C, the highest HK values (0.754 and 0.780 U/mg dry cell) were attained at pH 4.0. This result gives rise to increased glucose uptake by yeast at the level of the cytoplasmic membrane as observed by Bisson and Fraenkel *(4)*. The increased glucose uptake could also be observed from Fig. 2, because at pH 4.0 the glucose was completely consumed at t = 3 h, whereas at pH 5.0, this occurred after t = 4 h. Although the concentration of glucose was found to depend on invertase activity of the yeast, the formations of HK and invertase are uncoupled events. This observation is illustrated in Table 2 (pH 4.0 and 35 or 40°C), in which it is seen that high HK values occurred at the low values of invertase activity. Moreover, the same table indicates that the temperature influenced the invertase more sharply than the HK. Tests are being carried out to explain such results.

From the data presented, we have established suitable culture conditions for HK production by *S. cerevisiae*, which were 35 or 40°C, pH 4.0, and 4.0 mg O_2/L.

ACKNOWLEDGMENT

We are grateful to Mauro S. S. Candido for technical assistance.

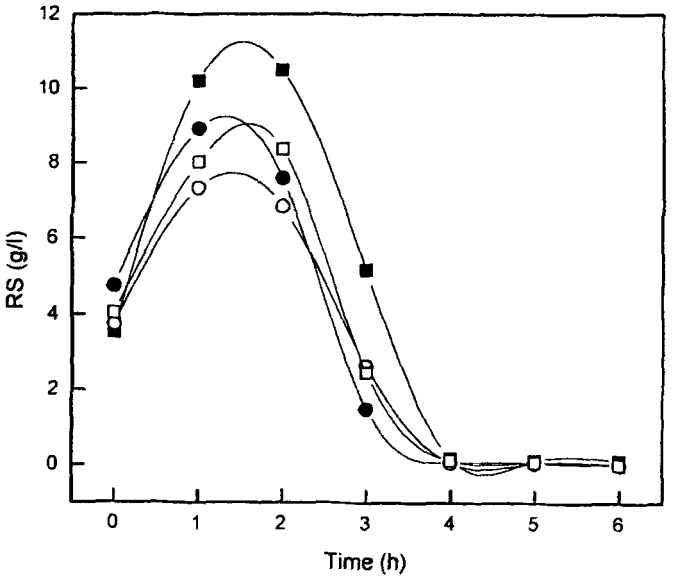

Fig. 1. Variation against time of RS concentration for batch cultures of *S. cerevisiae* carried out at pH 4.5, 35°C, and under the following DO (mg O_2/L): 0.2 (■), 2.0 (●), 4.0 (○), and 6.0 (□).

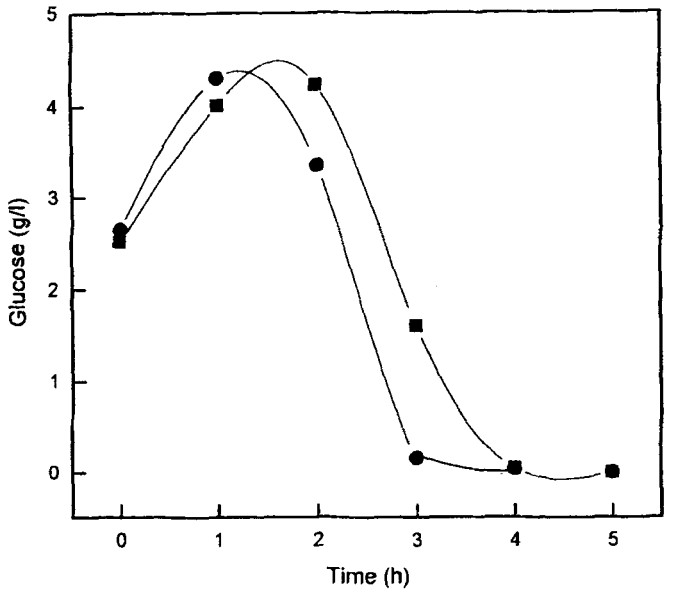

Fig. 2. Variation against time of the glucose concentration for batch cultures of *S. cerevisiae* carried out at 35 °C, DO = 4.0 mg O_2/L and at following pH: 4.0 (●) and 5.0 (■).

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