0273-2289/01/91–93/0605/$12.25

Effect of Agitation and Aeration on Production of Hexokinase by *Saccharomyces cerevisiae*

Daniel Pereira Silva,[1] Adalberto Pessoa, Jr.,[1] Inês-Conceição Roberto,[2] and Michele Vitolo*,[1]

[1]Department of Biochemical and Pharmaceutical Technology, Faculty of Pharmacy, University of São Paulo, Av. Prof. Lineu Prestes, 580, B.16. 05508-900, São Paulo/SP, Brazil, E-mail: michenzi@usp.br; and [2]DEBIQ-FAENQUIL, Lorena/SP, Brazil

Abstract

A batch culture of *Saccharomyces cerevisiae* for the production of hexokinase was carried out in a 5-L fermentor containing 3 L of culture medium, which was inoculated with cell suspension (about 0.7 g/L), and left fermenting at 35°C and pH 4.0. The aeration and agitation were adjusted to attain k_La values of 15, 60, 135, and 230 h^{-1}. The highest hexokinase productivity (754.6 U/[L·h]) and substrate-cell conversion yield (0.21 g/g) occurred for a k_La of 60 h^{-1}. Moreover, the formation of hexokinase and cell growth are coupled events, which is in accordance with the constitutive character of this enzyme. Hexokinase formation for $k_La > 60$ h^{-1} was not enhanced probably owing to saturation of the respiratory pathway by oxygen.

Index Entries: Hexokinase; *Saccharomyces cerevisiae*; agitation and aeration; fermentation; volumetric coefficient of oxygen transfer.

Introduction

Hexokinase (EC 2.7.1.1) is the first enzyme of glycolysis that catalyzes the phosphorylation of glucose into glucose 6-phosphate (G6P). The G6P, in turn, is a key intermediate for several pathways such as gluconeogenesis, shunt of pentoses, and glycogen metabolism. This enzyme is sensitive to the catabolic repression and has a role in the glucose uptake mechanism through the plasma membrane *(1)*.

Hexokinase, besides its role in the cell metabolism and importance to biochemical studies, is used in analytical methods to measure glucose, fructose, mannose, adenosine triphosphate (ATP), and creatin-kinase activity

*Author to whom all correspondence and reprint requests should be addressed.

(2); in the phosphorylation of pyranose and furanose analogs of glucose *(3)*; and in the wine and fruit juice industries for the detection of illegal sugar addition to the final products *(4)*.

Because hexokinase is a constitutive enzyme in all living cells, its production from a microorganism is linked to the amount of biomass obtained through a fermentative process. However, attaining a high amount of cells depends on the pH, temperature, components of the culture medium, and the availability of oxygen to the cells *(5)*. For a facultative microorganism such as *Saccharomyces cerevisiae*, oxygen has a crucial role in its overall metabolism, because it participates in the generation of energy, through the respiratory chain inside mitochondria, which is fundamental for attaining a significant specific growth rate. According to Gregory et al. *(6)*, there is a strong correlation among specific growth rate, oxygen transfer rate, and hexokinase production rate in yeasts. Although hexokinase could be attained from several microbial species, in Brazil, the use of *S. cerevisiae* as a source of this enzyme and other products *(7)* is practical owing to the large experience in handling this strain in industrial plants. In addition, coupling the yeast processing with the ethanol production probably should have a positive effect on distillery profits.

The present study deals with the influence of oxygen transfer rate, expressed as $k_L a$ (volumetric coefficient of oxygen transfer), on the production of hexokinase by *S. cerevisiae*.

Materials and Methods

Chemicals

Glucose 6-phosphate dehydrogenase, ATP, nicotinamide adenine dinucleotide phosphate (NADP), phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, G6P, glucose, and sucrose were obtained from Sigma (St. Louis, MO). Yeast extract and peptone were obtained from Difco (Detroit, MI). All other chemicals were of analytical grade.

Preparation of Inoculum

S. cerevisiae (isolated from pressed yeast cake) was maintained at 4°C in agar slant tubes containing 23.0 g/L of nutrient agar (Difco) and 1.0 g/L of glucose. The cells were transferred to 250-mL Erlenmeyer flasks containing 50 mL of autoclaved (121°C for 15 min) growth medium, and the culture was carried out in a shaker (New Brunswick, Edison, NJ) for 11 h at 35°C, agitation of 175 rpm, and initial pH of 4.5 (adjusted with 0.5 *M* H_2SO_4 or 0.1 *M* NaOH). The growth medium composition was as follows: 3.8 g/L of glucose, 28.5 g/L of sucrose, 3.0 g/L of yeast extract, 5.0 g/L of peptone, 2.4 g/L of $Na_2HPO_4 \cdot 12H_2O$, 0.075 g/L of $MgSO_4 \cdot 7H_2O$, and 5.1 g/L of $(NH_4)_2SO_4$.

Batch Fermentation

A volume of 0.45 L of inoculum (about 4.7 g of dry mass/L) was poured into a 5-L fermentor (NBS-MF 105, coupled with NBS dissolved oxygen

controller, DO-81; New Brunswick) containing 2.55 L of the culture medium: 3.0 g/L of yeast extract, 5.0 g/L of peptone, 20.0 g/L of glucose, 2.4 g/L of $Na_2HPO_4 \cdot 12H_2O$, 0.075 g/L of $MgSO_4 \cdot 7H_2O$, and 5.1 g/L of $(NH_4)_2SO_4$. The fermentation was carried out at 35°C and pH 4.0 (controlled automatically by adding 0.5 *M* H_2SO_4 or 0.1 *M* NaOH), and agitation and aeration were automatically adjusted to attain an initial k_La of 15 h^{-1} (0.7 vvm and 200 rpm), 60 h^{-1} (1.7 vvm and 400 rpm), 135 h^{-1} (2.3 vvm and 600 rpm), and 230 h^{-1} (2.3 vvm and 800 rpm). The pair agitation/aeration related to a desired initial k_La was fixed against distilled water and evaluated by using a dissolved oxygen controller (NBS-DO81; New Brunswick) associated with a galvanic dissolved oxygen probe (Mettler-Toledo, São Paulo, SP, Brazil). The initial k_La was calculated through the conventional Pirt's mathematical model *(8)*. To control the foam, a 10% (w/w) aqueous silicone emulsion (Dow Corning, FG-10; New York) was added dropwise. Samples were collected periodically to follow the variation in cell, glucose, and ethanol concentrations as well as hexokinase activity (after cell disruption).

Measurement of Cell, Glucose, and Ethanol Concentrations

One milliliter of fermenting medium was centrifuged (8.720 *g* for 10 min) and the supernatant discharged. Then, the cell cake was suspended in 50 mL of distilled water and the turbidity was measured with a spectrophotometer at 600 nm (Perkin-Elmer 552; Bethesda, MD). The cell concentration was obtained by comparing the optical density (OD) of cell suspension against a standard OD × dry cell mass curve.

Ten milliliters of fermenting medium was centrifuged (2.880 *g* for 10 min). The cell cake was stored at 4°C until submitted to disruption. In the supernatant, the glucose and ethanol concentrations were measured using a GOD/POD enzymatic kit (kit no. 02200; Laborlab, Guarulhos, São Paulo, SP, Brazil) and the reducing dichromate back-titration method *(9)*, respectively.

Measurement of Hexokinase Activity

The cell cake was suspended in 50 m*M* Tris-HCl buffer (pH 7.5) containing 5 m*M* $MgCl_2$, 10 m*M* β-mercaptoethanol, 2 m*M* aminocaproic acid, 1 m*M* phenylmethylsulfonyl fluoride, and 0.2 m*M* EDTA. Then, the cells were disrupted by submission to a vortex (Phoenix AP 56; São Paulo, SP, Brazil) in the presence of glass beads (diameter = 0.5 mm), and the mixture was maintained below 10°C all the time. The wet cell cake, glass beads, and Tris-HCl buffer were always mixed in a volumetric proportion of 1:1:1. Cell debris and glass beads were removed by centrifugation (2.880 *g* for 10 min) and the supernatant was collected. The hexokinase activity in the supernatant was measured through the continuous reduction of NADP at 30°C in a spectrophotometer (λ = 340 nm) as described by Bergmeyer *(2)*. One hexokinase unit was defined as the amount of enzyme catalyzing the reduction of 1 μmol of NADP/min under the assay conditions. Each determination

was made in triplicate and the standard deviation was 2%. No significant decrease in hexokinase activity was detected after storing the supernatant for 5 h on ice at room temperature.

Hexokinase production was calculated using the following equation:

$$P = (v/X_e) \times X \quad (1)$$

in which P = concentration of hexokinase (U/L_{medium}); v = hexokinase activity in the supernatant (U/mL); X_e = cell concentration in the suspension submitted to disruption (g_{cell}/mL); and X = cell concentration in the fermenting medium (g_{cell}/L_{medium}).

Calculation of Kinetic Parameters

The specific cell growth rate (μ_x), specific substrate consumption rate (μ_S), and specific hexokinase production rate (q_p) were defined as follows:

$$\mu_x = (1/X) \times dX/dt \quad (2)$$

$$\mu_S = (1/X) \times dS/dt \quad (3)$$

$$q_p = (1/X) \times dP/dt \quad (4)$$

The derivates dX/dt, dS/dt, and dP/dt were calculated according to the method proposed by Le Duy and Zajic *(10)*. The substrate-to-cell conversion factor ($Y_{X/S}$) and the substrate-to-hexokinase conversion factor ($Y_{HK/S}$) were calculated as the inclination obtained between the variation in cell concentration (ΔX) or hexokinase production (ΔP) to the glucose consumed (ΔS), respectively. The maximum enzyme (Pr_{HK}) and cell (Pr_X) productivities were calculated, respectively, as $\Delta P/\Delta t$ and $\Delta X/\Delta t$, in which Δt = interval of cultivation time.

Results and Discussion

From Fig. 1 we clearly observe that the k_La affected the hexokinase activity produced by the yeast. The highest hexokinase concentration, which was about 4.200 U/L_{medium}, occurred at a k_La of 60 h^{-1} after 7 h of fermentation. At this point, all glucose was consumed and the cell concentration was equal to 4.61 g/L (Figs. 2 and 3).

Nevertheless, at the extreme values of k_La employed (15 and 230 h^{-1}), the glucose concentration in the medium became negligible after 11 and 7 h, respectively (Fig. 2). Furthermore, the hexokinase concentration and cell concentration were markedly low for a k_La of 15 h^{-1} compared with any other k_La employed (Figs. 1 and 3). This result indicates that glucose consumption and hexokinase and cell production are coupled events and strongly depend on the aeration/agitation of the medium. In the present case, we can assume that the k_La of 60 h^{-1} guaranteed an adequate amount of dissolved oxygen in the medium to fine-tune the metabolic pathways related to growth and glucose conversion. This fact is clearly noted through

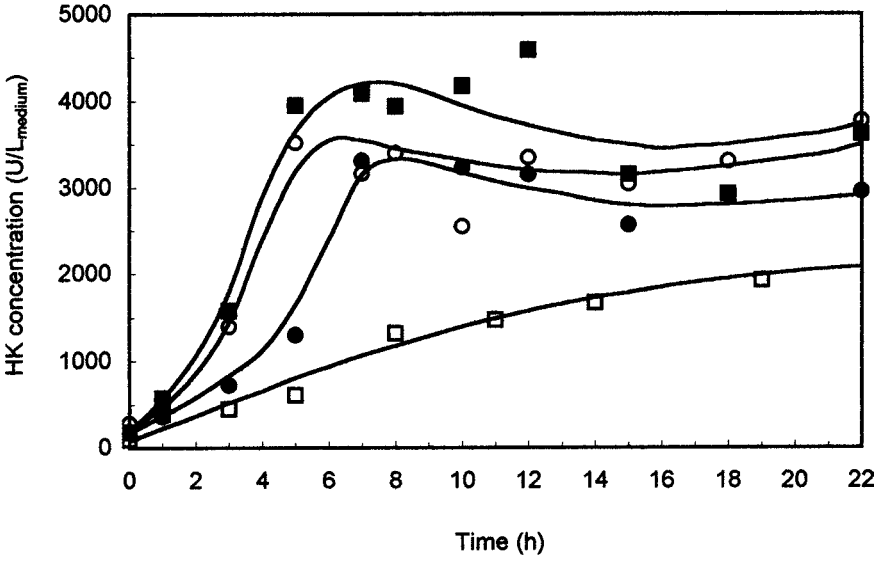

Fig. 1. HK production against time for *S. cerevisiae* grown under different k_La (h^{-1}) values: 15 (□), 60 (■), 135 (○), and 230 (●).

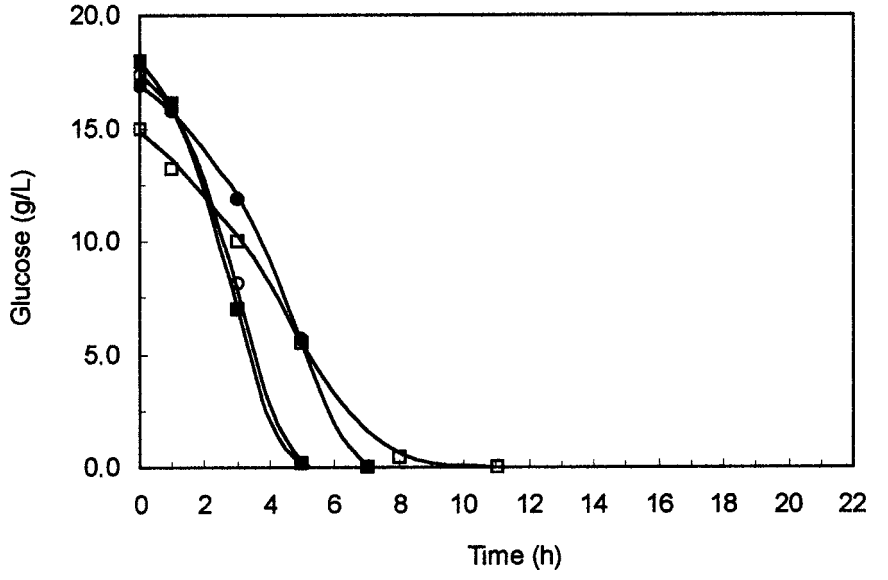

Fig. 2. Variation in glucose concentration against time for *S. cerevisiae* grown under different k_La (h^{-1}) values: 15 (□), 60 (■), 135 (○), and 230 (●).

the production parameters shown in Table 1, which at a k_La of 60 h^{-1}, $Y_{X/S}$ (0.21), $Y_{HK/S}$ (221.5 U/g_{glu}), Pr_X (0.69 $g_{cell}/[L{\cdot}h]$), and Pr_{HK} (754.6 $U/[L{\cdot}h]$) were the highest values attained. In addition, for this k_La the generation time (tg), calculated as proposed by Borzani *(11)*, was the lowest and equal to 1.8 h (Table 1).

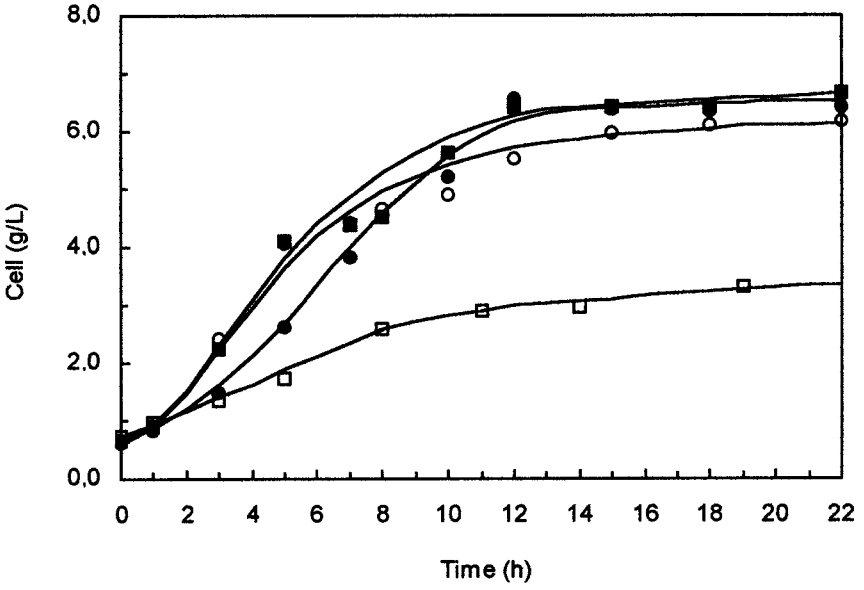

Fig. 3. Variation in cell concentration against time for *S. cerevisiae* grown under different k_La (h^{-1}) values: 15 (□), 60 (■), 135 (○), and 230 (●).

Table 1
Generation Time (tg), Glucose-to-Cell Conversion Factor ($Y_{X/S}$), Glucose-to-Hexokinase Conversion Factor ($Y_{HK/S}$), and Maximum Cell (Pr_X) and Hexokinase (Pr_{HK}) Productivities Related to Batch Fermentation of *S. cerevisiae* Conducted at Different k_La Values

	k_La (h^{-1})			
Parameter	15	60	135	230
tg (h)	4.3	1.8	1.8	2.5
$Y_{X/S}$ (g_{cell}/g_{glu})	0.14	0.21	0.21	0.19
$Y_{HK/S}$ (U/g_{glu})	82.8	221.5	192.9	175.8
Pr_X (g_{cell}/[L·h])	0.24	0.69	0.69	0.49
Pr_{HK} (U/[L·h])	300.5	754.6	647.0	445.7

From Table 1 it can also be seen that the parameters related to growth (tg, $Y_{X/S}$, and Pr_X) had the same values for either a k_La of 60 or 135 h^{-1}, whereas the hexokinase related parameters ($Y_{HK/S}$ and Pr_{HK}) differed about 15% at those k_La values. Most likely, for high k_La the intracellular ATP concentration is so high that it becomes an inhibitor of hexokinase biosynthesis *(12)*, although such a condition should favor cell growth. Values of k_La higher than 135 h^{-1} did not enhance cell growth owing probably to saturation of the respiratory pathway by oxygen.

It must be borne out that in all the experiments cell growth continued even when all the glucose initially present in the mash was consumed (Fig. 3).

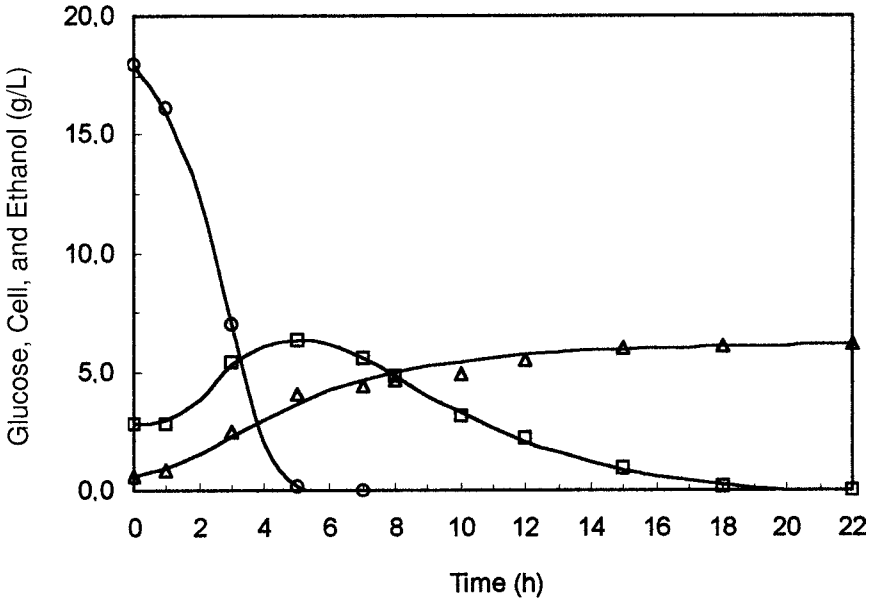

Fig. 4. Variation against time of glucose (○), cell (Δ), and ethanol (□) concentrations for *S. cerevisiae* grown at a k_La of 60 h^{-1}.

This can easily be understood if we consider that after glucose consumption another substance becomes a substrate to the yeast. In this case, a probable one should be ethanol, which accumulates into the medium because glucose has been taken up by the yeast (Fig. 4). According to Rose and Harrison *(13)* and Rehm and Reed *(14)*, the production of ethanol by *S. cerevisiae*, even under high aeration, is a characteristic of this specie of yeast. In other words, the Pasteur effect does not occur plentifully in *S. cerevisiae*.

Because hexokinase is a constitutive and growth-related enzyme, a viable process for its production would be a steady-state continuous culture, as already stated for other enzymes with the same characteristics, such as invertase *(15)*. In this case, according to Doin *(16)*, an estimation of the more suitable dilution rate (D) for the continuous process must be made from the data attained in batch. This can be accomplished by plotting the variation in growth rate (dX/dt) vs cell concentration (X) (Fig. 5). By setting 7 h of batch fermentation, as the period in which the highest hexokinase production was attained (Fig. 1), the correspondent cell concentration was 4.61 g/L (k_La = 60 h^{-1}). According to Doin *(16)*, the angular coefficient of the line drawn between the points (0, 0) and (4.61, 0.38) would be a reasonable estimation of D, which in this case should be about 0.08 h^{-1}. Of course, to produce hexokinase by direct extraction from residual distillery yeast is also an open opportunity for ethanol-producing countries (e.g., Brazil).

In short, to understand the data presented, we must take into account that the dependence of hexokinase on the availability of oxygen and cell growth derives from the fact that it is a constitutive-type enzyme as well as

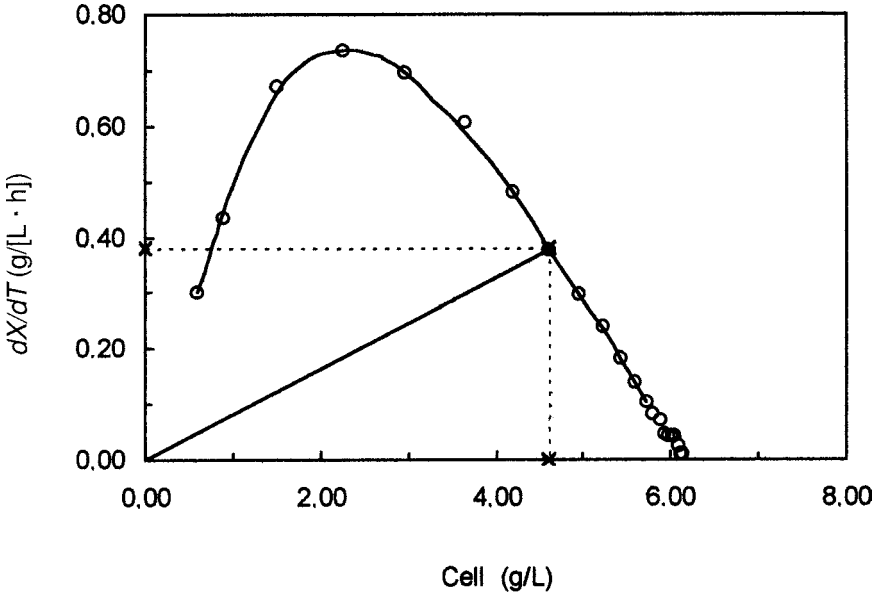

Fig. 5. Growth rate (dX/dt) vs cell concentration (X) for estimating the dilution rate (D) for a steady-state continuous culture based on the data obtained from a discontinuous culture carried out at an initial k_La of 60 h^{-1}.

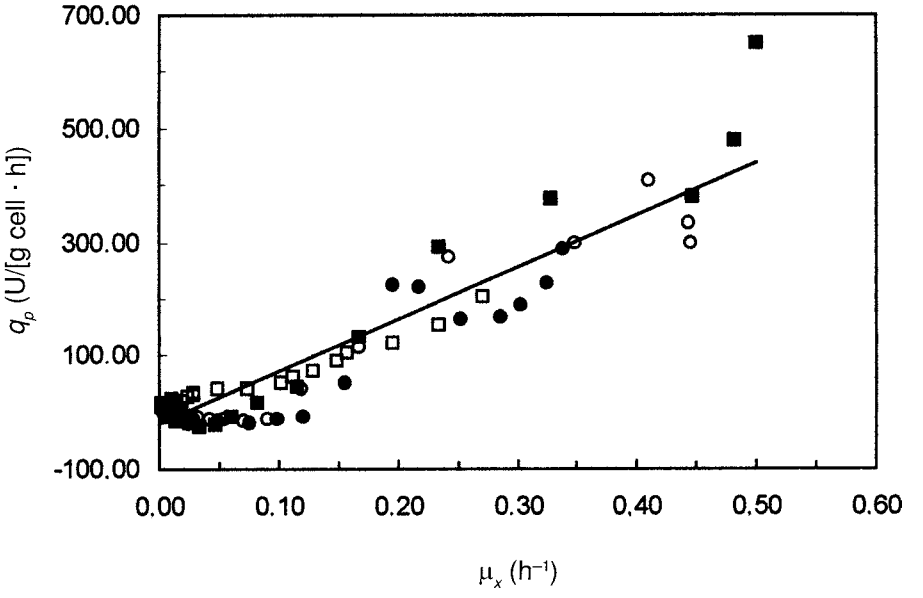

Fig. 6. Variation in HK specific production rate (q_p) against specific growth rate (μ_X) for *S. cerevisiae* grown under different k_La (h^{-1}) values: 15 (□), 60 (■), 135 (○), and 230 (●).

the G6P, the product of the hexokinase-catalyzed reaction, which is converted either in pyruvate (glycolysis pathway) or in 6-phosphoglucono lactone (6PL). The pyruvate is mainly directed to ATP generation (through

Krebs cycle and phosphorylative oxidation, both depending on oxygen), and 6PL is converted through the pentose pathway in sugars intimately related to growth and intracellular metabolism. However, a fraction of pyruvate is diverted to ethanol, which avoids the quantification of the correlation between the formation of hexokinase and cell growth by methods such as those of Luedeking and Piret *(17)* and Moser *(18)*. Figure 6, in which are plotted the data of all tests realized, shows a large points dispersion, an indication of the formation of at least one byproduct other than cells.

Conclusion

The data presented lead to two main conclusions. First, the high formation of hexokinase by *S. cerevisiae* occurred at a $k_L a$ of 60 h^{-1} after 7 h of batch culture. Second, because the formation of hexokinase is related to cell growth, a perspective should be to produce the enzyme through a continuous culture at a dilution rate of 0.08 h^{-1}, as estimated from the batch process.

Acknowledgments

We wish to thank Prof. Dr. José Abrahão Neto for valuable suggestions. D.P.S. acknowledges the financial support of FAPESP/São Paulo, Brazil, in the form of a Master of Science fellowship.

References

1. Kovak, L., Nelson, B. D., and Ernster, L. (1986), *Biochem. Biophys. Res. Comm.* **134(1),** 285–291.
2. Bergmeyer, H. U. (1984), *Methods of Enzymatic Analysis*, 3rd ed., Verlag Chimie, Weinheim, Germany.
3. Chenault, H. K., Mandes, R. F., and Hornberger, K. R. (1997), *J. Org. Chem.* **62,** 331–336.
4. Whitaker, J. R. (1991), *Food Enzymology*, Elsevier Applied Science, New York.
5. White, J. (1954), *Yeast Technology*, Chapman & Hall, London.
6. Gregory, M. E., Bulmer, M., and Bogle, I. D. L. (1996), *Bioproc. Eng.* **15(5),** 237–245.
7. Godfrey, T. and West, S. (1996), *Industrial Enzymology*, 2nd ed., MacMillan, London.
8. Wise, W. S. (1951), *J. Gen. Microbiol.* **5,** 167–177.
9. Joslyn, M. A. (1970), *Methods in Food Analysis*, 2nd ed., Academic, New York.
10. Le Duy, A. and Zajic, E. J. (1973), *Biotechnol. Bioeng.* **15,** 805–810.
11. Borzani, W. (1975), in *Engenharia Bioquímica*, vol. 3, Borzani, W., Lima, U. A., and Aquarone, E., eds., Universidade de São Paulo, São Paulo, pp. 168–184.
12. Garrett, R. H. and Grisham, C. M. (1995), *Biochemistry*, Saunders College Publishing, San Diego.
13. Rose, A. H. and Harrison, J. S. (1989), *The Yeasts*, 2nd ed., Academic, New York.
14. Rehm, H. J. and Reed, G. (1993), *Biotechnology*, 2nd ed., VCH, Weinheim, Germany.
15. Vitolo, M., Vairo, M. L. R., and Borzani, W. (1985), *Biotechnol. Bioeng.* **27,** 1229–1235.
16. Doin, P. A. (1975), in *Engenharia Bioquímica*, vol. 3, Borzani, W., Lima, U. A., and Aquarone, E., eds., Universidade de São Paulo, São Paulo, pp. 112–134.
17. Luedeking, R. and Piret, E. L. (1959), *J. Biochem. Microbiol. Technol. Eng.* **1,** 393–401.
18. Moser, A. (1985), in *Biotechnology: Fundamentals of Biochemical Engineering*, vol. 2, Rehm, H. J. and Reed, G., eds., VCH, Weinheim, Germany.