Journal of Industrial Microbiology (1995) 15, 75–79

Effect of pH, aeration and sucrose feeding on the invertase activity of intact *S. cerevisiae* cells grown in sugarcane blackstrap molasses

M Vitolo, MA Duranti and MB Pellegrim

Faculdade de Ciências Farmacêuticas, University of São Paulo, PO Box 66083, 05389-970, São Paulo, SP, Brazil

***S. cerevisiae* was grown in a blackstrap molasses containing medium in batch and fed-batch cultures. The following parameters were varied: pH (from 4.0 to 6.5), dissolved oxygen (DO) (from 0 to 5.0 mg O_2 L^{-1}) and sucrose feeding rate. When glucose concentration (S) was higher than 0.5 g L^{-1} a reduction in the specific invertase activity of intact cells (v) and an oscillatory behavior of v values during fermentation were observed. Both the invertase reduction and the oscillatory behavior of v values could be related to the glucose inhibitory effect on invertase biosynthesis. The best culture conditions for attaining *S. cerevisiae* cells suitable for invertase production were: temperature = 30° C; pH = 5.0; DO = 3.3 mg O_2 L^{-1}; (S) = 0.5 g L^{-1} and sucrose added into the fermenter according to the equations: $(V - V_o) = t^2/16$ or $(V - V_o) = (V_f - V_o) \cdot (e^{0.6t} - 1)/10$.**

Keywords: molasses; invertase activity; *S. cerevisiae*

Introduction

The yeast *Saccharomyces cerevisiae* is a useful source for several products of economic interest [5,8,9,18]. Among these is included invertase (EC.3.2.1.26), which has been largely used in confections and as a catalyst for inverted syrup production [6]. Recently, invertase has also been immobilized on sensors for continuous sucrose determination [1,10].

To produce *S. cerevisiae* cells suitable for invertase production, four main factors must be considered. First is a glucose inhibitory effect on invertase biosynthesis, which occurs at glucose concentrations higher than 2 g L^{-1} [11,15]. Secondly, the invertase activity of intact cells oscillates at intervals of about 2 h in either steady-state continuous or fed-batch cultures, as glucose concentrations range from 2 g L^{-1} to 5 g L^{-1} [11,17]. Recently, Rouwenhorst *et al* [13] reported similar results, observing that the oscillation was related to the yeast's budding cycle. Thirdly, the overall cost of invertase production could be diminished when molasses, a cheap by-product largely available in the sugar industry, was used for growing *S. cerevisiae*. Finally, the insertion of invertase within the cell wall and/or the adequate binding of polysaccharides to the wall structure could be affected by such culture conditions as pH.

The purpose of this research was to determine the best culture conditions to circumvent glucose repression and invertase activity oscillation, so as to obtain cells having high final invertase activity.

Correspondence: M Vitolo, Faculdade de Ciências Farmacêuticas, University of São Paulo, PO Box 66083, 05389-970, São Paulo, SP, Brazil
This work was supported by FAPESP
Received 15 August 1994; accepted 14 March 1995

Materials and methods

Inoculum preparation

S. cerevisiae (isolated from pressed yeast cake) was maintained on slant tubes containing (per liter) nutrient-agar (Difco, Detroit, MI, USA) 23.0 g and glucose 1.0 g. The cells were transferred to test tubes containing 2.5 ml of growth medium (5.0 g L^{-1} peptone; 10.0 g L^{-1} glucose and 3.0 g L^{-1} yeast extract; the pH was adjusted to 4.5 with HCl) and incubated at 33° C for 48 h. One tube was then used to inoculate 50 ml of molasses medium in a 250-ml Erlenmeyer flask, followed by incubation at 30° C for 22 h in a NBS Gyratory shaker (New Brunswick Scientific Co, Edison, NJ, USA) (frequency = 120 min^{-1}).

Molasses medium

The sugar cane blackstrap molasses medium was clarified as described previously [3]. After pH adjustment to 7.0 and dilution to reach glucose and sucrose concentrations of 8.5 g L^{-1} and 25.0 g L^{-1}, respectively, the clarified medium was sterilized at 120° C for 30 min. The medium was then supplemented with $(NH_4)_2SO_4$ (5.1 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.075 g L^{-1}) and $Na_2HPO_4 \cdot 12H_2O$ (2.4 g L^{-1}).

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

Batch fermentation

A volume of 0.45 L of inoculum (1.0 ± 0.2 g dry matter L^{-1}) was introduced into a 5-L bench fermenter (NBS-MF 200 coupled with an NBS-dissolved oxygen controller, DO-81) containing 2.55 L of sterilized molasses medium. The culture was then carried out under the following conditions: temperature of 30.0 ± 0.5° C; initial reducing sugars (S_o) 8.5 ± 2.1 g L^{-1}; initial total reducing sugars (S_o') 25.0 ± 3.0 g L^{-1}; impeller speed 500 min^{-1}, and 0.1 mg L^{-1} dimethylpolysiloxane (added dropwise as needed). The pH

and dissolved oxygen (DO) were varied from 4.0 to 6.5 and from 0 to 5.0 mg O_2 L^{-1}, respectively. Every hour, an aliquot of 10.0 ml of the culture medium was taken for analysis.

Fed-batch fermentation

A volume of 0.45 L of inoculum (1.0 ± 0.2 g dry matter L^{-1}) was introduced into a 5-L bench fermenter (NBS-MF 200 coupled with a DO-81 dissolved oxygen controller) containing 1.55 L sterilized molasses medium. The culture was then carried out under the following conditions: temperature $30.0 \pm 0.5°$ C; DO 3.3 mg O_2 L^{-1}; impeller speed 500 min^{-1} and pH 5.0. The culture was allowed to ferment batchwise for 3 h. A sucrose solution (30.0 g L^{-1}) was then fed into the reactor from the initial volume of 2 L up to a total volume of 3 L in both exponential and linear modes (filling time: 2 h or 4 h; Table 1). Once the feeding was completed, the fermentation was continued until sugar consumption was complete. Ten-milliliter aliquots of culture medium were taken at each hour for analysis. After sampling, 10.0 ml of sterile distilled water were added back to the fermenter.

In order to better determine the effect of pH on the final invertase activity of intact cells, additional fed-batch tests were carried out by adding sucrose according to an increasing linear mode (filling time = 4 h; Table 1), without aeration and at pH 4.0, 5.0, 6.0 and 7.0. Once the fermentation was terminated, the cells were separated by centrifugation ($10\,000 \times g$; 15 min), washed twice with distilled water, and the cell wall invertase was released as described below.

Measurement of glucose, ethanol and cell concentrations

Five milliliters of culture medium were filtered through a Millipore membrane (Millipore Division Products, Bedford, MA, USA) (pore diameter 0.45 μm). The cell concentration (X), expressed as g dry cell L^{-1}, was measured by drying the cell cake (105° C for 2 h). Glucose, sucrose and ethanol were determined in the filtrate. The glucose concentration (S) was measured by using a glucose analyzer equipped with a glucose oxidase probe (Technicon Auto-Analyser II, Technicon Instruments Co, Tarrytown, NY, USA). Sucrose and ethanol were measured as described previously [17]. Data related to ethanol concentration are not shown, because in all tests it was about 10 g L^{-1}.

Protein release from cell wall

Induced release of extracellular protein from the cell wall was achieved by suspending 100 mg of cells in 5 ml of a 50 mM potassium phosphate buffer (pH 7.0) containing 15 mM 2-mercaptoethanol and 15 mM dithiothreitol. The suspension was incubated for 2 h at 35° C, followed by centrifugation ($10\,000 \times g$; 10 min) at 4° C. The protein content in the supernatant fluid was measured according to Lowry *et al* [7].

Measurement of invertase activity

Invertase activity determinations (in duplicate) were carried out at 37° C in a mixture of 1.5 ml 0.01 M acetate–acetic acid buffer (pH 4.6), 2.5 ml 0.3 M sucrose solution and 0.5 ml invertase solution or cell suspension (both adequately diluted to assure that less than 2.0% of the sucrose present in the solution would be hydrolyzed). After 3 min, the hydrolysis was stopped by adding 1.0 ml of the Somogyi reagent [14], quickly followed by immersion in a boiling water bath for 10 min. The reducing sugar concentration (RS) was then measured as described previously [16].

One invertase unit (U) was defined as the amount of enzyme catalyzing the formation of 1 g of reducing sugars per hour at pH 4.6 and 37° C. Specific invertase activities were expressed as U mg^{-1} protein (soluble invertase) and U g^{-1} dry cells (insoluble invertase).

The cell suspension was prepared as follows: 5.0 ml of the culture medium were centrifuged ($3000 \times g$; 15 min); the sediment was washed with distilled water, centrifuged ($3000 \times g$; 15 min) and suspended in distilled water in order to obtain a known volume.

Results and discussion

Between 0 and 5 h incubation the specific invertase activity was lower than 10 U (g dry cells)$^{-1}$, but after 6 h it increased to approximately 100 U (g dry cells)$^{-1}$ (test carried out at pH = 5.0 and DO = 3.3 mg O_2 L^{-1}) (Figures 1 and 2). This result can be explained by assuming that the glucose inhibitory mechanism on invertase biosynthesis is

Table 1 Equations for sucrose feeding strategies used in fed-batch tests

Test	Mode	k[a]	T[b]	Integrated equations[c]
1	$F = F_o + kt$	0.25	2	$(V - V_o) = t^2/4$
2		0.13	4	$(V - V_o) = t^2/16$
3	$F = F_o - kt$	0.50	2	$(V - V_o) = t - t^2/4$
4		0.13	4	$(V - V_o) = t/2 - t^2/16$
5	$F = F_o \cdot e^{kt}$	0.60	2	$(V - V_o) = (V_f - V_o) \cdot (e^{0.6t} - 1)/2.32$
6		0.60	4	$(V - V_o) = (V_f - V_o) \cdot (e^{0.6t} - 1)/10$
7	$F = F_o/e^{kt}$	0.60	2	$(V - V_o) = (V_f - V_o) \cdot (1 - e^{-0.6t})/0.7$
8		0.60	4	$(V - V_o) = (V_f - V_o) \cdot (1 - e^{-0.6t})/0.9$

[a] k = time constant (h^{-1})
[b] T = fermentor filling-up-time (h)
[c] These equations were obtained by assuming that $V = V_f$ when $t = T$, and integrating as previously reported [19]

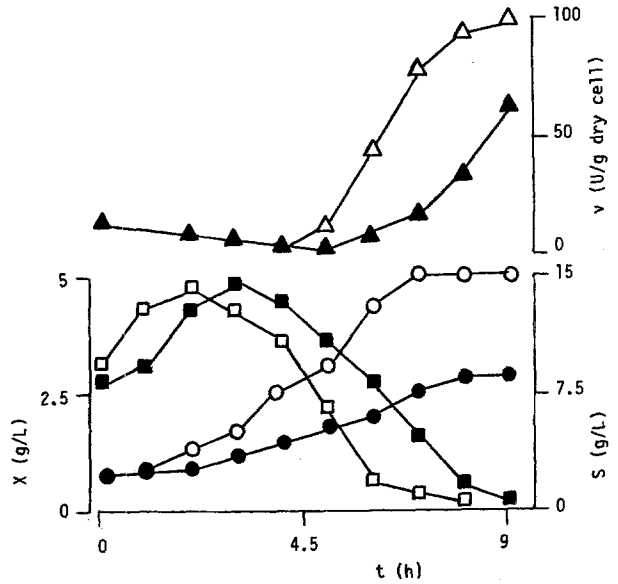

Figure 1 Specific invertase activity (▲, △), cell (●, ○) and reducing sugar (■, □) concentration for batch tests carried out at 30° C and pH 5.0. The dark and open symbols refer to DO = 0 and DO = 3.3 mg O_2 L^{-1}, respectively

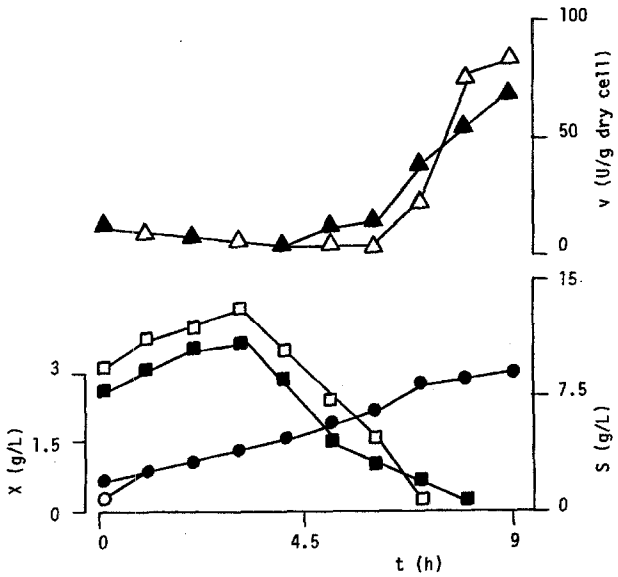

Figure 2 Specific invertase activity (▲, △), cell (●, ○) and reducing sugar (■, □) concentration for batch tests carried out at 30° C without aeration. The dark and open symbols refer to pH 4.0 and 5.5, respectively

switched off when the glucose concentration in the medium reaches a limit value [15]. In the literature, the upper limit established for glucose concentration is 2.0 g L^{-1} [11]. However, the molasses medium studied here contained a concentration about three times lower (Figures 1 and 2, after 6 h). Furthermore, the glucose concentration in the medium also varied during the fermentation, increasing up to 3 h, and diminishing afterwards (Figures 1 and 2). This could result from sucrose hydrolysis catalyzed by the invertase present in the yeast cell wall [4], associated with the low cell concentration in the fermenter during that step (1.0 g L^{-1}).

The effect of pH and dissolved oxygen on the growth of *S. cerevisiae* can be observed through the variation of substrate consumption/dry cell ratio ($Y_{x/s}$ = yield factor), which ranged from 0.08 to 0.24 (Table 2). The yield factor was defined as the conversion of total reducing sugars, measured as glucose, into cells [2].

In the tests with no aeration, $Y_{x/s}$ was not affected by pH (Table 2). As shown by Weitzel *et al* [20], *S. cerevisiae* has an intracellular buffering capability, which could explain why the pH of culture—within the range studied—did not significantly affect the overall intracellular sugar conversion. Furthermore, it can be assumed that the external pH interferes with nutrient uptake and/or the budding capability of the cells (maintained at DO near zero) leading to a defined pH value (4.5 in the present case) under which the specific growth rate reaches a maximum value (0.63 h^{-1}).

Nevertheless, the final invertase activity of intact cells at pH 5.5 was about 20% higher than at pH 4.0 (Figure 2). The specific invertase activity, solubilized from yeast cell wall, varied around 48%, depending on the pH of the culture from which the cells were harvested (Table 3). Such a result can be due to modifications in the tertiary and quaternary structures of invertase as a consequence of the external pH, leading to an anomalous insertion of the enzyme inside the cell wall network [12]. Of course, perturbations at the level of the invertase biosynthesis pathway cannot be totally disregarded.

In all cases the specific invertase activity was higher than the specific growth rate (Table 2). This constitutes evidence that the enzyme was not a limiting factor for cell growth, leading to the assumption that the invertase activity of intact cells and the conversion of substrate into cells are

Table 2 Specific invertase activity, conversion of substrate into cells ($Y_{x/s}$) and specific growth rate for all batch tests

	Dissolved oxygen (mg L^{-1})		
pH	0	1.67	3.33
4.0	6.12[a]	4.92	4.86
	0.32[b]	0.27	0.32
	0.10[c]	0.11	0.07
4.5	2.46[a]	4.98	3.36
	0.63[b]	0.30	0.29
	0.09[c]	0.14	0.12
5.0	3.84[a]	3.24	3.30
	0.16[b]	0.29	0.36
	0.08[c]	0.14	0.24
5.5	3.84[a]	4.20	2.70
	0.20[b]	0.39	0.32
	0.10[c]	0.16	0.18
6.0	9.06[a]	8.16	3.90
	0.22[b]	0.41	0.39
	0.10[c]	0.16	0.19
6.5	6.42[a]	7.20	1.80
	0.27[b]	0.37	0.30
	0.10[c]	0.17	0.18

[a]Specific invertase activity [g reducing sugars h^{-1}(g cells)$^{-1}$]
[b]Specific growth rate (h^{-1})
[c]Conversion of substrate (as glucose) into dry cells

Table 3 Specific invertase activity and protein concentration in the supernatant phase after induced release of cell wall proteins from cells harvested from fed batch tests

pH	Protein (mg ml^{-1})	Specific invertase activity[a] [U (mg protein)$^{-1}$]
4.0	0.98 ± 0.01	0.41 ± 0.05
5.0	1.10 ± 0.02	0.62 ± 0.09
6.0	1.00 ± 0.02	0.32 ± 0.04
7.0[b]	—	—

[a]This activity was determined in samples containing 1 mg ml^{-1} protein
[b]Data releated to pH 7.0 were not acquired, because cell aggregation prevented proper sampling for the invertase activity measurement

unrelated events. Therefore, invertase attached to the cell wall acts like a typical immobilized enzyme [19].

Invertase production (Figure 3a), cell productivity (Figure 3b) and generation time (Figure 3c) (calculated, according to Borzani [2]), for all batch tests are presented in Figure 3. The highest invertase production ($P = 500$ U L^{-1}) and cell productivity ($P_x = 0.6$ g dry cells L^{-1} h^{-1}) were obtained for the culture carried out at pH = 5.0 and DO = 3.3 mg O_2 L^{-1}. Under such conditions the generation time was 1.5 h, leading to a highly budded cell population in the fermenter as observed by microscopic analysis. This result is in agreement with Rouwenhorst *et al* [13], who found a correlation between the degree of budding and invertase activity.

The overall fermentation time can be lessened if glucose inhibition is circumvented by narrowing the period during which the specific invertase activity reduces markedly (Figures 1 and 2). In order to determine more precisely the

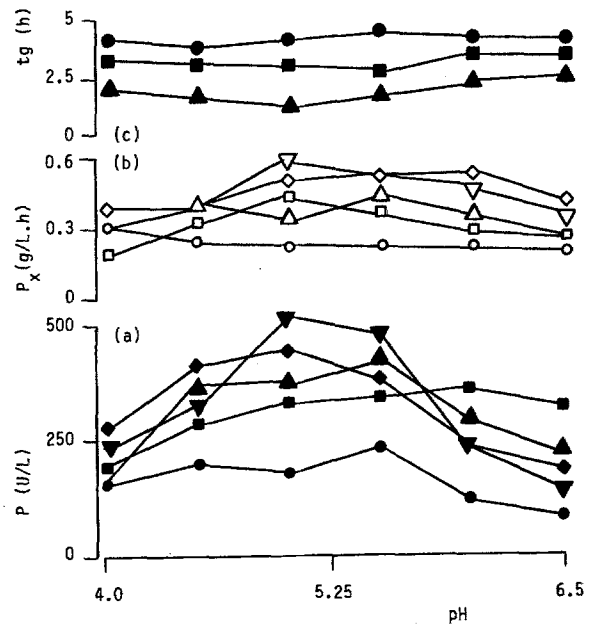

Figure 3 Data from batch tests. (3a): Variation of invertase production against pH at DO (mg O_2 L^{-1}): 0 (●), 0.83 (■), 1.67 (▲), 3.33 (▼) and 5.0 (◆). (3b):Variation of cell productivity against pH at DO (mg O_2 L^{-1}): 0 (○), 0.83 (□), 1.67 (△), 3.33 (▽) and 5.0 (◇). (3c): Variation of generation time against pH at DO (mg O_2 L^{-1}): 0 (●), 0.83 (■), 1.67 (■), 3.33 (▲) and 5.0 (■)

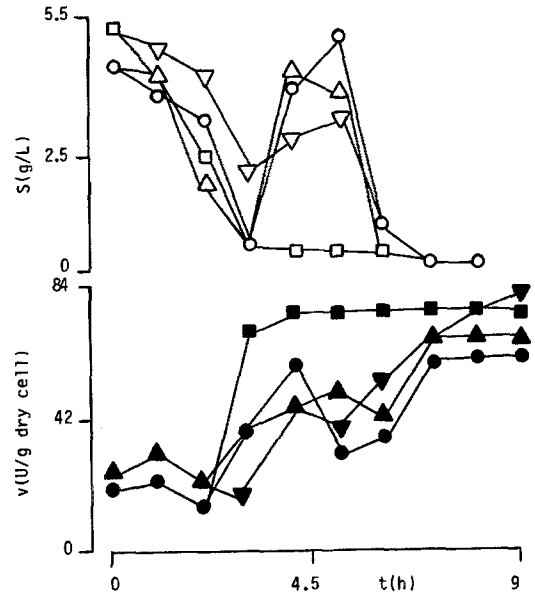

Figure 4 Specific invertase activity (solid symbols) and reducing sugar concentration (open symbols) related to fed-batch tests 1 (▲, △), 2 (■, □), 3 (●, ○) and 4 (▼, ▽), in which sucrose was added according to a linear mode

upper limit of glucose concentration, several fed-batch tests were carried out under different sucrose feeding strategies (Table 1).

Regarding test numbers 2 and 6, (Figures 4 and 5) addition of sucrose to the fermenter by either an increasing exponential or increasing linear mode (filling time = 4 h in both cases), the glucose concentration in the medium after 2 h was constant and equal to 0.5 g L^{-1}. Thus, at such a concentration the glucose inhibitory effect is eliminated. Moreover, working at that glucose level, the whole fermentation time can be reduced by 50% and oscillation of the specific invertase activity (observed in all tests except in

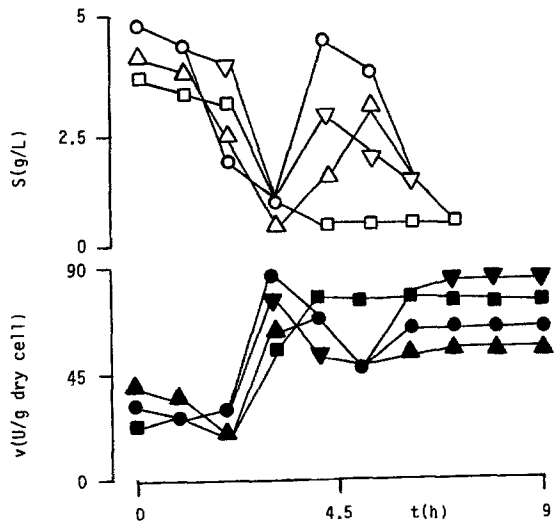

Figure 5 Specific invertase activity (solid symbols) and reducing sugar concentration (open symbols) related to fed-batch tests 5 (▲, △), 6 (■, □), 7 (●, ○) and 8 (▼, ▽), in which sucrose was added according to an exponential mode

Table 4 Cell productivity (Px), generation time (tg) and invertase production (P) for fed-batch tests

Test (no)	Px ($g\ L^{-1}\ h^{-1}$)	tg (h)	P ($U\ L^{-1}$)
1	0.22 ± 0.01	2.5	491 ± 8
2	0.22 ± 0.01	1.5	550 ± 7
3	0.23 ± 0.02	4.2	374 ± 9
4	0.23 ± 0.01	3.0	400 ± 11
5	0.24 ± 0.03	2.2	515 ± 8
6	0.23 ± 0.01	1.9	537 ± 7
7	0.23 ± 0.01	3.4	442 ± 8
8	0.23 ± 0.02	3.7	467 ± 7

numbers 2 and 6) was not observed. These data indicate that the oscillation of v during fed-batch culture and glucose inhibition are related phenomena. Such a result is in agreement with Rouwenhorst *et al* [13], who observed oscillation of invertase activity in tests carried out at a glucose concentration of 2.0 $g\ L^{-1}$.

Finally, highest invertase production (about 550 $U\ L^{-1}$) was attained when sucrose was added according to an increasing linear mode (Table 4, filling time = 4 h; test number 2). Furthermore, cell productivity was not affected by the sucrose feeding rate (the variation coefficient was lower than 3%), whereas the generation time depended on the manner by which sucrose was added.

Acknowledgements

To Dra Marina Franco for technical support.

References

1 Barlikova A, J Svorc and S Miertus. 1991. Hybrid biosensor for the determination of sucrose. Analyt Chim Acta 247: 83–87.
2 Borzani W. 1975. Continuous fermentation. In: Biochemical Engineering (Borzani W, AL Urgel and E Aquarone, eds), p 112, Edgard Blucher Ltd, São Paulo.
3 Borzani W, RE Gregori and MLR Vairo. 1977. Some observations on oscillatory changes in the growth rate of *S. cerevisiae* in aerobic continuous undisturbed culture. Biotechnol Bioengin 19: 1363–1374.
4 Gunther K, N Schulke, FX Schmid and R Jaenicke. 1992. Stability quaternary structure, and folding of internal, external, and core-glycosylated invertase from yeast. Protein Sci 1: 120–131.
5 Kelly M. 1983. Yeast extract. In: Industrial Enzymology (Godfrey T and J Reichelt, eds), p 457, MacMillan, Hong-Kong.
6 Layman PL. 1986. Industrial enzymes: battling to remain specialities. Chem Engin News 15: 11–14.
7 Lowry OH, NV Rosenbrough, RV Farr and RV Randall. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–271.
8 Mahmoud W, AHM El-Sayed and RW Coughlin. 1990. Production of L-phenylacetylcarbinol by immobilized yeast cells. Biotechnol Bioengin 36: 47–63.
9 Naoshima Y, T Nishiyama and Y Munakata. 1989. Bioreduction of ethyl 3-Oxobutanoate with immobilized baker's yeast in organic-water solvent systems. Chem Lett 8: 1517–1518.
10 Park JK, HS Ro and HS Kim. 1991. A new biosensor for specific determination of sucrose using an oxidoreductase of *Z. mobilis* and invertase. Biotechnol Bioengin 38: 217–223.
11 Patkar A and JH Seo. 1992. Fermentation kinetics of recombinant yeast in batch and fed-batch cultures. Biotechnol Bioengin 40: 103–109.
12 Reddy AV, R Marcoll and F Maley. 1990. Effect of oligossaccharides and chloride on the oligomeric structures of external, internal and deglycosylated invertase. Biochemistry 29 (10): 2482–2487.
13 Rouwenhorst RJ, AA Van der Baan, AW Scheffers and JP Van Dijken. 1991. Production and localization of beta-fructosidase in asynchronous and synchronous chemostat cultures of yeasts. Appl Environ Microbiol 57: 557–562.
14 Somogyi M. 1952. Notes on sugar determination. J Biol Chem 195: 19–23.
15 Trumbly RJ. 1992. Glucose repression in the yeast *S. cerevisiae*. Molec Microbiol 6: 15–21.
16 Vitolo M and W Borzani. 1983. Measurement of invertase activity of cells of *S. cerevisiae*. Analyt Biochem 130: 469–470.
17 Vitolo M, W Borzani and MLR Vairo. 1985. Invertase activity of intact cells of *S. cerevisiae* growing on sugarcane molasses I. Steady-state continuous culture tests. Biotechnol Bioengin 27: 1229–1235.
18 Vitolo M and MT Yassuda. 1991. Effect of sucrose concentration on the invertase activity of intact yeast cells. Biotechnol Lett 13: 53–56.
19 Vitolo M, JCM Carvalho, MA Duranti and M Breda. 1991. Invertase activity of intact yeast cells harvested from fed-batch ethanol fermentation of sugarcane blackstrap molasses. Biomass and Bioenergy 1(5): 301–304.
20 Weitzel G, U Pilatus and L Rensing. 1987. The cytoplasmic pH, ATP content and total protein synthesis rate during heat-shock protein inducing treatments in yeast. Experiment Cell Res 170: 64–79.