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CIRCUMVENTING THE GLUCOSE REPRESSION ON INVERTASE ACTIVITY OF BAKER'S YEAST BY USING FED-BATCH FERMENTATION

Prof. Michele Vitolo*

School of Pharmaceutical Sciences, University of São Paulo, Brazil.

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***Corresponding Author**
Prof. Michele Vitolo
School of Pharmaceutical Sciences, University of São Paulo, Brazil.

ABSTRACT

An important subject in invertase production by baker's yeast is overcome the repression caused by glucose present in the mash during fermentation. The aim was pursued by carrying out the fermentation in fed-batch mode. A 5L-bench fermenter containing 1.55L molasses medium was inoculated with 0.45L of inoculum (1.0g dry matter/L). The culture was carried out at 30°C, pH 5.0, 3.3mg O_2/L and impeller speed of 500 min^{-1}. Sugarcane molasses (30 g/L sucrose) was fed into the fermenter from the initial volume of 2L up to a total volume of 3.0L in both linear and exponential modes at filling time of 4h. For comparison, a batch test was carried out at the same conditions (except sucrose which all amount was added at the beginning of the fermentation). The main results showed that the catabolic repression was eliminated at glucose concentration below 0.5 g/L and the fermentation conducted in increasing linear or exponential feeding mode. Such fed-batch processes allowed to diminish the whole fermentation time by 50% in comparison with the batch process.

KEYWORDS: Yeast, invertase, molasses, *Saccharomyces cerevisiae*, fed-batch fermentation.

INTRODUCTION

Baker's yeast (*Saccharomyces cerevisiae*) – largely used in bakery and ethanol production - is a useful source for several products of economic interest. Among these invertase is included (E.C.3.2.1.26), which has been used in confectionary industry, in inverted sugar production, in enzyme electrodes for sucrose determination and as medicine, associated or

not with other enzymes, for antimicrobial and antioxidant actions.[1][2] Moreover, the cell wall localization of invertase enables the intact yeast cells to be a natural immobilized system, which would be used in the continuous sucrose hydrolysis for attaining inverted syrup.[3]

Previously it was shown that invertase formation by *S. cerevisiae*, grown in molasses by batch fermentative process, was subjected to catabolite repression, which occurred at glucose concentrations higher than 2 g/L.[4][5]

A manner to minimize the glucose catabolite repression effect could be avoiding that glucose concentration in the mash surpass 2g/L during fermentation. This aim could be achieved through fed-batch fermentation, in which the fermenter is fed with glucose or sucrose step by step. However, the fermenter feeding must follow a determined addition law (linear or exponential both at decreasing or increasing mode) – considering the filling time of fermenter and the constant time of addition[3] -, which lead to a glucose amount in the mash below the repression limit. Thereby, the purpose of this work was to establish the fed-batch feeding mode under which the catabolite repression could be circumvented.

MATERIAL AND METHODS

Inoculum preparation

S. cerevisiae (isolated from commercial pressed yeast) was maintained on slant tubes containing (per liter) nutrient-agar (Difco, Detroit, MI, USA) 23.0g and glucose 1.0g. The cells were transferred to test tubes containing 2.5mL of growth medium (5.0 g/L peptone; 10.0g/L glucose and 3.0g/L yeast extract); the pH was adjusted to 4.5 with 1M HCl) and incubated at 33^{o}C for 48h. One tube was then used to inoculate 50mL of molasses medium in a 250mL conical flask, followed by incubation at 30^{o}C for 22h 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, diluted (glucose = 8.5g/L and sucrose = 30.0g/L) and sterilized as described previously.[4] The medium was supplemented with $(NH_4)_2SO_4$ (5.1g/L), $MgSO_4.7H_2O$ (0.075g/L) and $Na_2HPO_4.12H_2O$ (2.4g/L). In all the tests, the pH of the medium during fermentation was maintained at the desired value by automatic addition of 1M NaOH or 0.5M H_2SO_4.

Fed-batch fermentation

5L-bench fermenter (NBS-MF 200 coupled with a DO-81 dissolved oxygen controller) containing 1.55L molasses medium was inoculated with 0.45L of inoculum (1.0g dry matter/L). The culture was carried out at 30^{o}C, pH 5.0, 3.3mg O_2/L and impeller speed of 500 min^{-1}. The culture was allowed to ferment batchwise for 3h, aiming the propagation of the inoculum. A sucrose solution (30.0g/L) was then fed into the fermenter from the initial volume of 2L up to a total volume of 3.0L in both linear and exponential modes at filling time of 4h (Table 1). Once the feeding was completed, the fermentation was continued until sugar consumption was complete. Ten milliliter aliquots of mash were taken at each hour for analysis. After sampling, 10mL of sterile distilled water were added back to the fermenter.

In order to compare the fed-batch results attained a 9h-batch fermentation was carried out under the same fed-batch culture conditions. Details of the process were fully described previously.[4]

Table 1: Equations for Sucrose Feeding Strategies Used in Fed-Batch Tests.

Test	Mode[a]	k	Integrated equations[b]
I	$F = F_o + k.t$	0.13	$(V - V_o) = t^2/16$
II	$F = F_o - k.t$	0.13	$(V - V_o) = t/2 - t^2/16$
III	$F = F_o.e^{kt}$	0.60	$(V - V_o) = (V_f - V_o).(e^{0.6t} - 1)/10$
IV	$F = F_o.e^{-k.t}$	0.60	$(V - V_o) = (V_f - V_o).(1 - e^{-0.6t})/0.9$

[a]F (feeding rate; L/h); F_o (initial feeding rate; L/h); k (addition constant; linear mode: L/h^2; exponential mode: h^{-1}); t (addition time; h). [b] $(V - V_o)$ (volume added; L); $(V_f - V_o)$ (total volume of sucrose solution to be added; in our case 1L).

Measurement of glucose, sucrose and cell concentrations

Five milliliters of mash were filtered through a 0.45µm-Millipore membrane (Millipore Division Products, Bedford, MA, USA). The cell concentration, expressed as g dry cell per Liter, was measured by drying the cell cake (105^{o}C for 2h). Glucose and sucrose were determined in the filtrate. The glucose concentration was measured by using a glucose analyzer equipped with a glucose oxidase probe (Technicon Auto-analyzer II, Technicon Instruments Co, Tarrytown, NY, USA). Sucrose was measured as described previously.[4]

Measurement of invertase activity

Invertase activity measurements (in duplicate) were carried out at 37^{o}C in a mixture of 1.5mL 0.01M acetate-acetic acid buffer (pH 4.6), 2.5 mL 0.3M sucrose solution and 0.5 mL cell

suspension (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's alkaline solution, quickly followed by immersion in a boiling water bath for 10 min. The reducing sugars concentration, expressed as glucose, was determined as described above.

One invertase unit (U) was defined as the amount of enzyme catalyzing the formation of 1g of reducing sugars per hour at pH 4.6 and 37^{o}C. Specific invertase activity was expressed as U/g dry cell.

The cell suspension was prepared as follows: 5.0mL of the mash were centrifuged (3000xg; 15 min); the sediment was washed with distilled water, centrifuged (3000xg; 15 min) and suspended in distilled water in order to obtain a known volume.

RESULTS AND DISCUSSION

In all the experiments, samples were taken at each hour in which the cell concentration (X), invertase activity (v) and glucose concentration (S) were measured. The results were shown in Table 2.

The collected data were plotted in graphics correlating each measured parameter with the fermentation time (Figures 1-3). The specific cell growth rate (μ), generation time (t_g), cell productivity (P_x) and invertase production (P) were calculated through equations 1-4 (Table 3).

$$LnX - LnX_i = \mu.t \qquad \text{(Eq. 1)}$$

$$t_g = Ln2 \div \mu \qquad \text{(Eq. 2)}$$

$$P_x = (X_f - X_i) \div t \qquad \text{(Eq. 3)}$$

$$P = (v_f.X_f - v_i.X_i) \qquad \text{(Eq. 4)}$$

Where X = cell concentration in the medium (g/L); X_i = initial cell concentration (g/L); X_f = final cell concentration (g/L); v_i = initial specific invertase activity (g glucose/g dry cell.h); v_f = final specific invertase activity (g glucose/g dry cell.h); μ = specific cell growth rate (h^{-1}); t_g = generation time (h); P_x = cell productivity (g/L.h); P = invertase production (U/L).

The cell concentration in fed-batch tests varied between 0.73 and 2.70 g/L regardless of the feeding mode, whereas in batch the test varied significantly (Table 2). The distinct behavior occurs due to the non-uniform availability of nutrients (mainly carbon source) for yeast

subjected at different culture regimens. In fed-batch regimen the carbon source was offered to the cell more parsimoniously than in the batch, so that metabolic pathways not directly related to growing are favored, such as formation of trehalose, glycogen, amongst other compounds.[6] This point can be envisaged if it is considered that the final specific invertase activity of yeast cultured in batch regimen (101 g glucose/g cell.h) is about 22% higher than the average enzyme activity of yeast (78.8 g glucose/g cell.h) grown in fed-batch regimen. Therefore, lesser available amount of carbon source for yeast under fed-batch leads to the diminution of the overall enzyme activity required.

Table 2: Variation of specific invertase activity (v; g glucose/gcell.h), cell (X; g/L), reducing sugars (S; g/L) concentrations during batch and fed-batch fermentations.

Test	Variables	Fermentation time (h)									
		0	1	2	3	4	5	6	7	8	9
	X	0.70	0.82	1.20	1.63	2.50	3.16	4.27	4.96	4.99	5.00
Batch	S	10.6	12.9	13.0	12.7	10.8	6.30	1.80	0.74	0.69	0.50
	v	11.8	13.3	7.92	5.70	3.30	6.18	4.45	78.0	94.2	101
	X	0.75	0.80	1.14	1.44	1.69	1.99	2.26	2.54	2.73	2.74
I	S	4.54	4.01	2.44	0.56	0.51	0.46	0.42	0.39	0.02	0
	v	27.2	28.1	23.4	68.4	70.8	75.6	78.0	78.6	79.2	80.0
	X	0.78	0.83	1.11	1.45	1.83	1.85	2.41	2.62	2.79	2.82
II	S	5.06	4.64	4.12	2.19	2.64	2.95	1.02	0.26	0.31	0
	v	23.2	25.7	19.2	18.1	46.7	38.3	49.1	75.6	80.4	81.6
	X	0.78	0.88	1.18	1.59	1.84	2.17	2.23	2.64	2.83	2.70
III	S	4.84	3.90	2.31	0.72	0.50	0.32	0.28	0.14	0.09	0,03
	v	25.9	33.6	24.2	63.0	70.2	76.2	80.0	75.0	75.5	75.0
	X	0.73	0.88	1.26	1.47	1.74	2.11	2.44	2.77	2.64	2.70
IV	S	4.47	4.09	2.16	0.43	2.84	2.31	0.36	0.28	0.10	0,04
	v	38.5	32.8	27.2	81.6	57.2	47.2	76.2	82.2	92.4	78.6

Figure 1 shows that the specific invertase activity of yeast cultured in batch regimen diminished from 11.8 to 4.45 g glucose/g cell.h up to 6h, following a sharp increasing until the end of the fermentation (101 g glucose/g cell.h). This behaviour is correlated with the variation of glucose concentration in the mash, which below 2 g/L seems to push the increasing of invertase activity. This result represents a typical catabolite repression mechanism, which is often observed in enzymes located at the yeast cell wall.[4] In terms of invertase production, the catabolite repression must be circumvented because having an invertase activity reduction of about 62% during 67% overall duration of fermentative process is inadmissible. Thereby, yeast culture through fed-batch mode in which the glucose

concentration in the mash can be maintained below the inhibitory value below 2 g/L- value observed in discontinuous culture -becomes a valuable alternative.

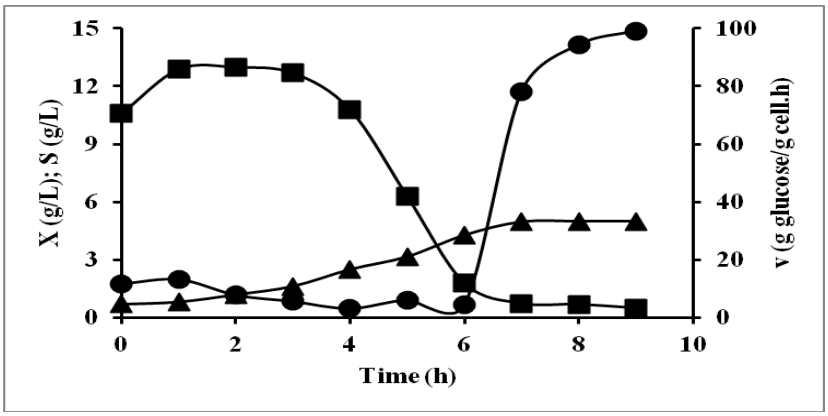

Figure 1. Specific invertase activity (●), cell (▲) and glucose (■) concentration for batch test.

From Figures 2 and 3 it can be seen that the increasing linear and exponential modes, related to tests I and III, respectively, allowed maintaining the glucose concentration in the mash after 2h constant and equal to 0.5 g/L, as well as the specific invertase activity reached a maximal value of about 80 g glucose/g cell.h at 3h-9h interval of the process. Thereby, at glucose concentration ≤ 0.5 g/L the catabolite repression effect is circumvented. Moreover, working at that glucose level, the whole fermentation time can be reduced by 50% and oscillation of the specific invertase activity (observed in tests II and IV) was not observed. These data indicate that the oscillation of specific invertase activity during fed-batch culture and glucose inhibition are related phenomena. Oscillation of invertase activity in synchronous chemostat cultures of yeasts was observed when the glucose concentration was 2.0 g/L.[7]

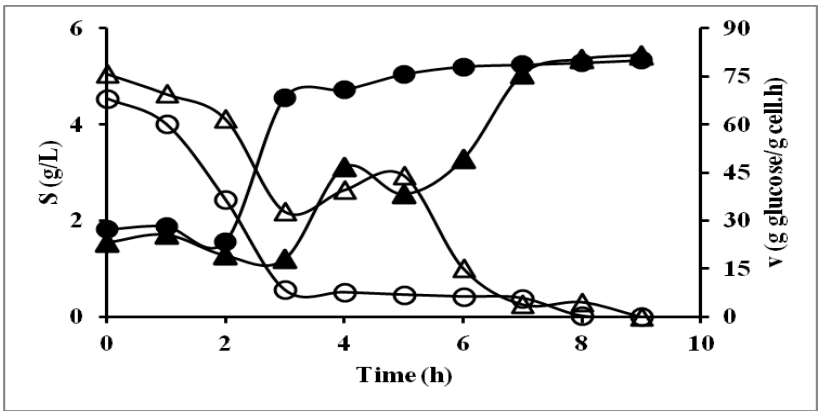

Figure 2. Specific invertase activity (solid symbols) and glucose concentration (open symbols) related to fed-batch tests I (●, o) and II (▲, Δ), in which sucrose was added according to a linear mode.

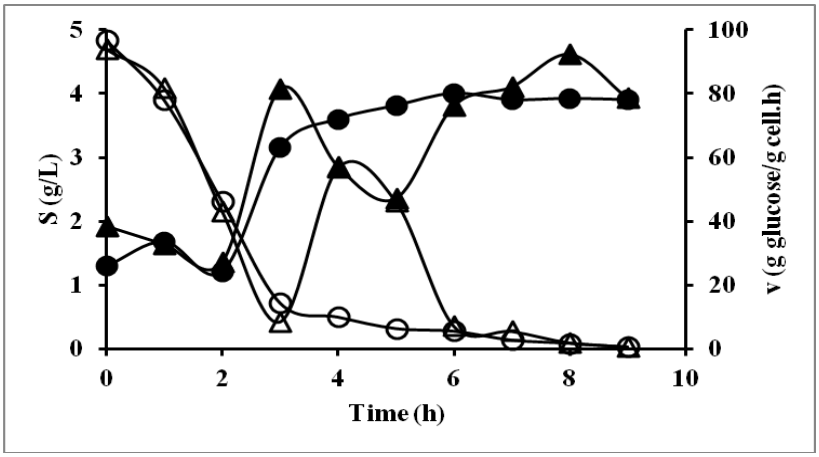

Figure 3: Specific invertase activity (solid symbols) and glucose concentration (open symbols) related to fed-batch tests III (●, o) and IV (▲, Δ), in which sucrose was added according to a exponential mode.

Finally,the highest invertase production (212 U/L) was attainned when sucrose was added according to an increasing linear mode (Table 3; test I). Furthermore, cell productivity practically did not change with the sucrose feeding mode, whereas the generation time depended on the manner which sucrose was added to the fermenter. Thus, tests II (decreasing linear mode) and IV (decreasing exponential mode) had generation times twofold higher than tests I and III (Table 3).

Table 3: Cell productivity (P_x), generation time (t_g) and invertase production (P) for fed-batch tests.

Test (n.)	P_x (g/L.h)	μ (h^{-1})	t_g (h)	P (U/L)
I	0.22	0.46	1.50	199
II	0.23	0.23	3.01	212
III	0.21	0.36	1.93	182
IV	0.22	0.19	3.85	184

CONCLUSION

The presented data led to conclude that the catabolic repression promoted by glucose can be eliminated when glucose concentration in the mash is not over 0.5 g/L and the fermentation is conducted in fed-batch mode. Moreover, fed-batch processes, mainly the increasing linear and exponential modes, allowed to diminish the whole fermentation time by 50% in comparison with the batch process. Besides, the invertase production, specific growth rate and generation time depended on the type of sucrose feeding employed, whereas the cell productivity was independent of the manner how the sucrose was added.

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