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Fed-Batch Production of Glucose 6-Phosphate Dehydrogenase Using Recombinant *Saccharomyces cerevisiae*

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Abstract

The strain *Saccharomyces cerevisiae* W303-181, having the plasmid YEpPGK-G6P (built by coupling the vector YEPLAC 181 with the promoter phosphoglycerate kinase 1), was cultured by fed-batch process in order to evaluate its capability in the formation of glucose 6-phosphate dehydrogenase (EC.1.1.1.49). Two liters of culture medium (10.0 g/L glucose, 3.7 g/L yeast nitrogen broth (YNB), 0.02 g/L L-tryptophan, 0.02 g/L L-histidine, 0.02 g/L uracil, and 0.02 g/L adenine) were inoculated with 1.5 g dry cell/L and left fermenting in the batch mode at pH 5.7, aeration of 2.2 vvm, 30°C, and agitation of 400 rpm. After glucose concentration in the medium was lower than 1.0 g/L, the cell culture was fed with a solution of glucose (10.0 g/L) or micronutrients (L-tryptophan, L-histidine, uracil, and adenine each one at a concentration of 0.02 g/L) following the constant, linear, or exponential mode. The volume of the culture medium in the fed-batch process was varied from 2 L up to 3 L during 5 h. The highest glucose 6-phosphate dehydrogenase activity (350 U/L; 1 U = 1 μmol of NADP/min) occurred when the glucose solution was fed into the fermenter through the decreasing linear mode.

Index Entries: Fed-batch; glucose 6-phosphate dehydrogenase; glucose; recombinant strain; *Saccharomyces cerevisiae;* fermentation.

Introduction

Glucose 6-phosphate dehydrogenase (G6PD) (EC.1.1.1.49), a constitutive enzyme present in all cells, is largely used as reagent in clinical diagnostic and chemical analysis methods *(1)*. Among all G6PD available sources, the *Saccharomyces cerevisiae* deserves special attention because it is a nonpathogenic microorganism, its biochemistry and genetic mechanisms have been intensely studied throughout the 20th century and it is intensely used in industry (ethanol distilleries, bakery, for instance) *(2)*. To circumvent the metabolic control on the formation of G6PD by the

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wild *S. cerevisiae* strain, a quite common event for a constitutive enzyme, genetically modified yeast was used, aiming to overproduce the enzyme. Lojudice et al. *(3)* attained the recombinant strain *S. cerevisiae* W303-181 by introducing the plasmid YEpPGK-G6P in the wild yeast strain. The plasmid, in turn, was constructed from the vector YEPLAC 181—a mutant allele of *LEU2* gene of wild *S. cerevisiae* fully described *(4)*—combined with the promoter phosphoglycerate kinase 1.

Lojudice et al. *(3)* evaluated the growing and G6PD formation capability by the engineered yeast through a batch culture carried out at 35°C, pH 4.0, aeration of 2.3 vvm, and in a medium constituted of glucose (20.0 g/L), peptone (5.0 g/L), yeast extract (3.0 g/L), $Na_2HPO_4 \cdot 12H_2O$ (2.4 g/L), $MgSO_4 \cdot 7H_2O$ (0.075 g/L), and $(NH_4)_2SO_4$ (5.1 g/L). The G6PD specific activity attained under these conditions was equal to 0.300 U/g_{cell}. Indeed, the authors directed their efforts preferentially to the retention of the plasmid YEpPGK-G6P and not to the formation of G6PD by the modified yeast. However, it was observed through batch culture that the G6PD formation was stimulated at glucose concentration in the medium higher than 7.0 g/L *(5)*. Such a result prompted to carry out fed-batch tests in order to establish the correlation between G6PD formation and glucose concentration. The fed-batch culture, as well known, allows maintaining the substrate concentration in the medium inside well-defined interval, provided that the substrate addition into the fermenter obeys a defined feeding strategy *(6)*. From the literature consulted (*[7,8]*, among others), studies have demonstrated that the optimization of the fed-batch culture can improve the gene expression by the vector. Moreover, an initial study of the *S. cerevisiae* W303-181 grown in fed-batch cultivations was performed by glucose addition according to exponential mode and the results obtained demonstrated that the high-level of G6PD formation was related to cell growth and the plasmid not excreted to the extracellular medium *(9)*. Nevertheless, more studies are necessary to establish the best fed condition including nutrient and feeding strategies. Thereby, the present work aims to evaluate the effect of the feeding strategies on the G6PD formation by *S. cerevisiae* W303-181 cultured through a fed-batch process.

Materials and Methods

Microorganism and Maintenance Culture Medium

S. cerevisiae W303-181 was kindly assigned by the Chemical Institute of University of São Paulo through Dra. Carla Columbano Oliveira. Lojudice et al. *(3)* described all the genetic engineering techniques used in modifying the yeast strain as well as the culture medium (CM) for maintaining its viability. The maintenance culture medium (MCM) consisted of 20.0 g/L glucose, 7.4 g/L YNB, 15.0 g/L agar, 0.02 g/L L-histidine, 0.02 g/L L-tryptophan, 0.02 g/L uracil, and 0.02 g/L adenine.

Subcultivation was done every month at 30°C for 48 h. After that the plates (stock cultures) were stored at 4°C.

Preparation of the Inoculum

A loopful of the stock culture was transferred to tubes containing 5 mL of CM (the MCM without agar) and incubated at 30°C for 24 h. The content of one tube was then transferred to 500-mL Erlenmeyer flask with 95 mL of CM and incubated on a rotary shaker (NBS Gyratory Shaker, New Brunswick Scientific Co., Edison, NJ) at 150 rpm at 30°C for 18 h. A total of sixteen 500-mL Erlenmeyer flasks were used (total volume = 1.6 L).

Fed-Batch Culture

A volume of 0.10 L of inoculum (total cell mass of 3.0 g on dry basis) was introduced into a 5-L bench fermenter (NBS-MF 105, coupled with a DO-81 dissolved oxygen controller) containing 1.9 L of CM. The glucose solution (20.0 g/L), the micronutrients solution (constituted of L-histidine, L-tryptophan, uracil, and adenine each one at a concentration of 0.020 g/L), or the solution containing glucose (20.0 g/L) and micronutrients (each one at 0.020 g/L) was then fed into the reactor (in pulses at intervals of 30 min) from the initial volume (V_0) of 2 L up to a final volume (V_f) of 3 L through constant, linear, or exponential mode. In all tests the fermenter filling-up-time was set at 5 h. The culture was carried out at 30°C, pH 5.7, impeller speed of 400 rpm, and aeration of 2.2 vvm. Once the feeding was completed, the fermentation was continued until the glucose concentration in the medium was negligible. Fifteen milliliter aliquots of the CM were collected at each hour for analysis. After sampling, 15.0 mL of sterile water was added back to the fermenter. A total of ten tests were realized (Table 1). On the feeding plan, the feed rates were well controlled and reproducible. Air was bubbled for 15 min before adding the inoculum. The pH of the medium was maintained at the chosen value by the controlled addition of 0.5 *M* NaOH or 0.5 *M* H_2SO_4. The foam was controlled, whenever needed, by addition of drops of dimethylpolysiloxane.

Cell Disruption

At each hour a sample of 5 mL of the fermenting broth was centrifuged (4100*g*, 20 min) and the pellet was rinsed twofold with 5 mL of distilled water. The pellet was suspended in 50 m*M* TRIS-HCl buffer (pH 7.5), 5.0 m*M* $MgCl_2$, 0.2 m*M* ethylenediamine tetra acetic acid, 10.0 m*M* β-mercaptoethanol, 2.0 m*M* aminocaproic, and 1.0 m*M* phenylmethylsulfonyl fluoride. The cells were disrupted through stirring with 0.5-mm glass beads for 12 min (dry cell matter/mass glass beads ratio of 1 : 300). Cell debris and glass beads were removed by centrifugation (4100*g*, 20 min). The supernatant was used to measure enzyme activity.

Table 1
Identification of the Tests Regarding the Nutrient Fed and the Feeding Strategy Used

Test (no.)	Nutrient fed	Feeding mode[a]	Equations[b]	k (L/h^2)	F_0 (L/h)
1	G[c]	$F = F_0$	$(V - V_0) = 0.2 \cdot t$	–	0.2
2	M[d]	$F = F_0$	$(V - V_0) = 0.2 \cdot t$	–	0.2
3	G	$F = F_0 - k \cdot t$	$(V - V_0) = 0.4 \cdot t - 0.04 \cdot t^2$	0.08	0.4
4	M	$F = F_0 - k \cdot t$	$(V - V_0) = 0.4 \cdot t - 0.04 \cdot t^2$	0.08	0.4
5	G	$F = F_0 + k \cdot t$	$(V - V_0) = 0.04 \cdot t^2$	0.08	0
6	M	$F = F_0 + k \cdot t$	$(V - V_0) = 0.04 \cdot t^2$	0.08	0
7	G	$F = F_0 . e^{-k \cdot t}$	$0.92 \cdot (V - V_0) = (V_0 - V_f) \cdot (e^{-0.5 \cdot t} - 1)$	0.5	0.54
8	M	$F = F_0 . e^{-k \cdot t}$	$0.92 \cdot (V - V_0) = (V_0 - V_f) \cdot (e^{-0.5 \cdot t} - 1)$	0.5	0.54
9	G	$F = F_0 . e^{k \cdot t}$	$11.2 \cdot (V - V_0) = (V_f - V_0) \cdot (e^{0.5 \cdot t} - 1)$	0.5	0.045
10	M	$F = F_0 . e^{k \cdot t}$	$11.2 \cdot (V - V_0) = (V_f - V_0) \cdot (e^{0.5 \cdot t} - 1)$	0.5	0.045
11	G/M[e]	$F = F_0 - k \cdot t$	$(V - V_0) = 0.4 \cdot t - 0.04 \cdot t^2$	0.08	0.4

For all tests the fermentor filling-up time was 5 h.
[a]Feeding mode:F (reactor feeding rate [L/h]), F_0 (initial reactor feeding rate [L/h]), k (feeding constant [L/h^2]), and t (time [h]).
[b]These equations were obtained by assuming that $V = V_f$ when $t = T$ and integrating as previously reported *(14)*.
[c]G, glucose.
[d]M, micronutrients (solution containing L-tryptophan, L-histidine, uracil, and adenine each one at concentration of 0.02 g/L).
[e]G/M, glucose plus micronutrients (solution containing 20 g/L of glucose and 0.02 g/L of each micronutrient cited).

Analytical Procedures

Measurement of Cell Concentration

At each hour a sample of 10 mL of the fermenting broth was filtered through a microfiltration membrane (Millipore® HAWP04700), and the cell cake was rinsed twofold with distilled water. The cell concentration, expressed as gram of dry matter/L was determined *(6)*. The variation coefficient of this technique was 4.49%.

Measurement of Glucose Concentration

The glucose concentration was determined as the total reducing sugars present in the sample of the fermenting broth *(6)*. The variation coefficient related to this method was 3.86%.

Measurement of G6PD

The determination of G6PD activity was made through the continuous reduction of NADP at 30°C in a spectrophotometer (Beckman DU650, $\lambda = 340$ nm) (Beckman Coulter, Fullerton, CA) as described by Bergmeyer *(10)*. One G6PD unit (U) 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 variation coefficient was less than 1%. Throughout the work the G6PD activity was expressed as U/L (A) and U/g dry cell (U/g_{cell}) (A_{cell}).

Detection of Cell Viability

Samples of 1.0 mL were taken each 2 h or 3 h during the culture for detecting the cell viability. The approaches considered as viability criteria were:

- **Methylene blue colorant absorption by the cells—**the blue colored cells were counted through a conventional Neubauer chamber ($1/400 \times 0.100$ mm^3). One percent of colored cells was considered as the superior limit for high-viability.
- **Cell cultivation in MCM——**the appearance of a characteristic pink color in the solid medium was taken as indication of viability. The intensity of coloration was visually compared with a standard stock culture of *S. cerevisiae* W303-181, prepared as already described.

In all tests the realized yeast cells remained viable during the cultivation (through the methylen blue adsorption criteria) as well as retained the plasmid (through the criteria of cultivation in MCM).

Kinetic Parameters

The generation time (t_g) was calculated as proposed by Vitolo et al. *(11)*. The cell (P_x) and G6PD (P_{G6PD}) productivity were calculated, respectively, as the ratio of cell mass (ΔMx) and enzyme activity (ΔA) to the cultivation time

Table 2
Parameters Related to the Fed-batch Cultures of *S. cerevisiae* W303-181

Test (no.)	t_g (h)	t_{max} (h)	A (U/L)	A_{cell} (U/g_{cell})	P_{G6PD} (U/L·h)
1	Nd	6	190	65	32
2	13	8	240	50	30
3	4	5	350	65	70
4	3	10	200	105	20
5	7	12	270	61	23
6	5	10	140	31	14
7	6	12	234	36	20
8	8	10	300	45	30
9	5	5	300	48	60
10	11	5	185	32	37
11	15	7	96	22	14

Generation time (t_g), duration of the fermentation process (t_{max}), highest G6PD activity (A), and G6PD specific activity (A_{cell}). Nd, not determined.

(Δt). The whole duration of the fermentation process (t_{max}) was set as the period of time elapsed between the beginning of fermenter feeding and the completion of glucose consumption.

Results and Discussion

The high G6PD activities attained were 350 U/L and 300 U/L, respectively, in tests 3 (glucose fed; t_{max} = 5 h; linear decreasing feeding mode) and 8 (micronutrients fed; t_{max} = 10 h; exponential decreasing feeding mode) (Table 2). Taking into account the enzyme formed in test 3 was about 18% higher than in test 8, the glucose was better than the micronutrients as the fed substrate for stimulating the G6PD formation by the recombinant yeast. This result is understandable considering that glucose is the source of carbon and energy utilized by the yeast for growing as well as fulfilling the necessities of its overall metabolism, whereas the micronutrients are funneled preferentially to specific metabolic pathways (for instance, biosynthesis of nucleic acids and proteins).

From Figs. 1 and 2 it can be seen that the feeding mode as well as the type of nutrient fed influenced the G6PD specific activity (A_{cell}) formed by *S. cerevisiae* W303-181 along the culture. When the substrate fed through the linear decreasing strategy was the glucose (test 3), the highest A_{cell} (about 65 U/g_{cell}) was reached at t = 2 h remaining constant till the end of the culture (Fig. 1). However, when the substrate fed through the same strategy was the micronutrient solution (test 4), the highest A_{cell} (about 105 U/g_{cell}) was reached between t = 3 h and t = 4 h, followed by a reduction of about 52% till the end of the fermentation (Fig. 2). Probably

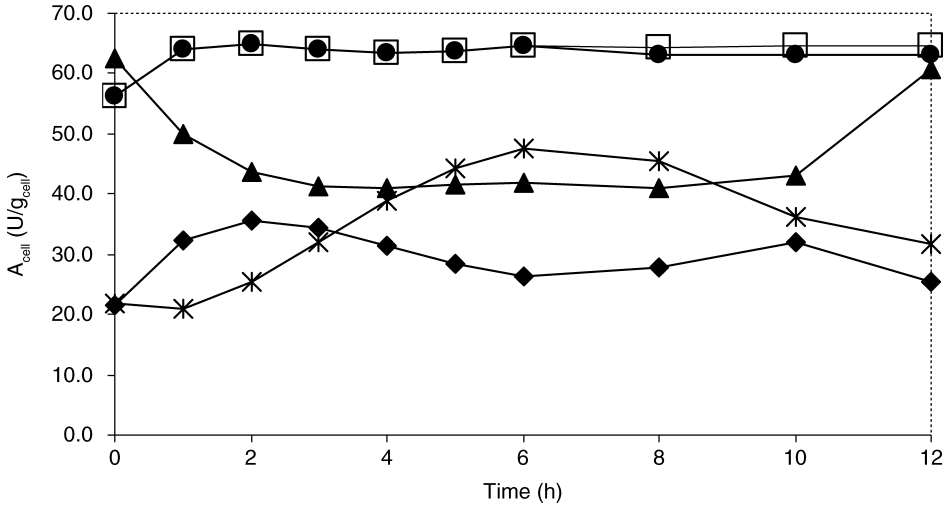

Fig. 1. Variation of G6PD specific activity (A_{cell}) during the culture time (t), when glucose was the fed nutrient. Tests 1 (●), 3 (□), 5 (▲), 7 (◆), and 9 (✳).

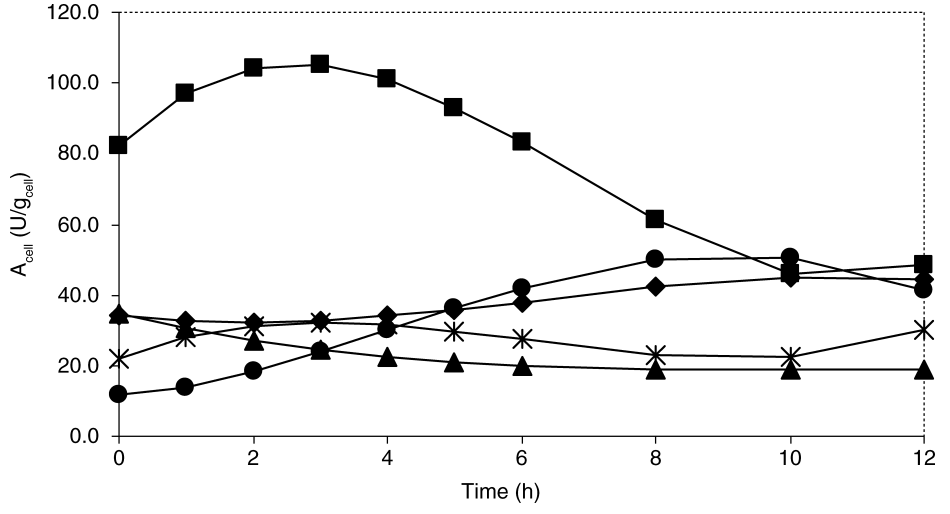

Fig. 2. Variation of G6PD activity (A_{cell}) during the culture time (t), when micronutrients were the fed substrate. Tests 2 (●), 4 (■), 6 (▲), 8 (◆), and 10 (✳).

an amino acid/nucleotide limitation occurred in test 4, insofar as the generation time (t_g) of 3 h was 25% lower than that of test 3 (t_g = 4 h) (Table 2). As the *S. cerevisiae* W303-181 has in the nucleus one additional G6PD codifying gene, the dependence on amino acids and nucleotides can be more conspicuous.

The best G6PD activities attained through the decreasing feeding strategy (linear or exponential) might be because of the availability of glucose or micronutrients present in the medium to the cells. In any

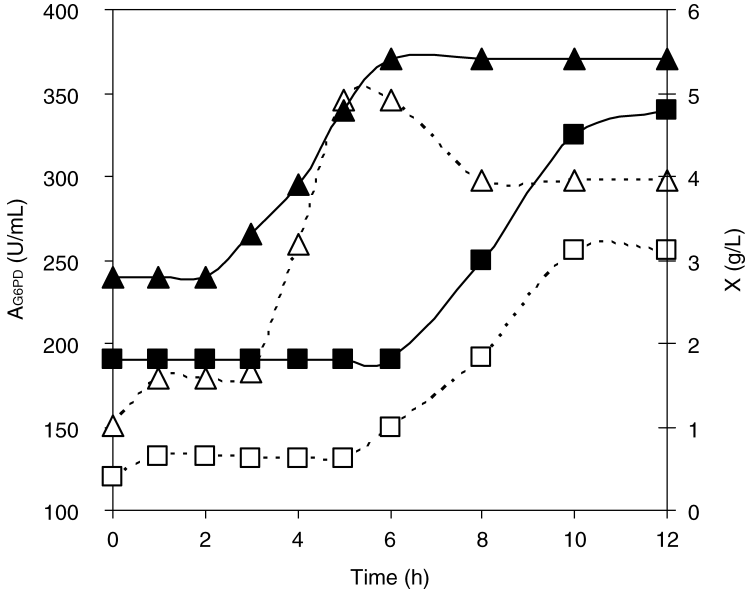

Fig. 3. Variation of G6PD activity concentration during the fed-batch culture of *S. cerevisiae* W303-181 in which glucose ([▲] A_{G6PD} and [■] X) and micronutrients ([△] A_{G6PD} and [□] X) were added according to the decreasing linear mode.

decreasing feeding strategy the amount of substrate available to the cells is always high at the beginning of the culture, diminishing afterwards. From the correspondent feeding equations (Table 1) [$(V–V_0) = 0.4 \cdot t – 0.04 \cdot t^2$ and $0.92 \cdot (V – V_0) = (V_0 – V_f) \times (e^{-0.5\,t} –1)$] the starting volumes for glucose (linear decreasing strategy) and micronutrients (exponential decreasing strategy) are 190 and 241 mL, respectively. According to Brown et al. *(12)* the cell growth must be stimulated over the enzyme formation in the beginning of culture, so that after a period of time (say about 2 h, in the present work) the *G6PD* gene can be derepressed and the enzyme synthesis proceeds. Such a behavior is attained as the linear or exponential feeding strategy is used.

Figure 3 shows clearly that the formation of a constitutive enzyme is linked to the cell mass formed, a well-known phenomenon *(13)*. However, what is notorious is the different behavior presented by *S. cerevisiae* W303-181 regarding the type of substrate added into the CM. When the fed substrate was glucose the cell and G6PD formation ceased after $t = 5$ h. Moreover, the final enzyme activity diminished about 14%. In the case of feeding with the micronutrients solution, a pronounced lag phase occurred before the formation of cell and G6PD. Because of the amino acid/nucleotide limitation the strain might need an adaptation period for its metabolism to the culture conditions. This might explain the oscillation of A_{cell} along the time observed when comparing tests 3, 4, and 11 (Fig. 4) with the fermenter being fed, respectively, with glucose,

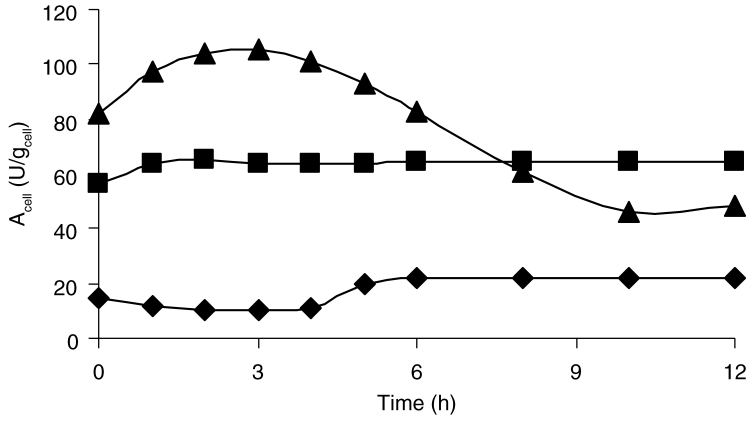

Fig. 4. Variation of G6PD specific activity (A_{cell}) during the culture time (t), when glucose (■), micronutrients (▲), and both (glucose and micronutrients) (◆) were the fed nutrients added according to the decreasing linear mode.

micronutrients, and both nutrients (glucose and micronutrients) according to the decreasing linear mode. The A_{cell} in test 11 was 66% less than obtained in test 3 (fed with glucose) and 79% less than test 4 (fed with micronutrients). Moreover, test 11 presented the lowest G6PD specific activity (A_{cell} = 22 U/g_{cell}) when comparing with the nutrients fed isolated in all feeding modes.

In spite of the best fed-batch culture conditions for the G6PD, formation by *S. cerevisiae* W303-181 must be still established. The result attained in test 3 (P_{G6PD} = 70 U/L·h) (Table 2) is sevenfold higher than that found in the best batch fermentation process (P_{G6PD} = 10.5 U/L·h), which was carried out under the same conditions, except the feeding rate *(5)*. However, the P_{G6PD} was 47% lower than that found by Miguel et al. *(9)*, whose conditions differed on glucose concentration (5.0 g/L instead of 10.0 g/L as used in the present work), concentration of micronutrients (8.0 µg/L instead of 20 µg/L), and the feeding rate (0.2/h instead of 0.5/h). Through the fed-batch approach it is possible to couple the end of the substrate addition with the high formation of a desired product, which can reduce costs when the process is operated in an industrial plant *(12)*. In the present work the condition $T = t_{max}$ occurred in tests 3, 9, and 10 (Table 1).

Finally, having alternatives for G6PD production is quite important for an enzyme-importing country (like Brazil), because this particular enzyme has a large use in accurate diagnostic tests (mainly, enzyme-immunoassay technique) for the identification of several diseases, most of them endemic in developing countries. Nevertheless, the cost of the CM utilized in this work for the growth of the recombinant strain is very high, because expensive micronutrients are required. However, significant cost reduction might be attained using a low expensive CM constituted of sugarcane blackstrap molasses and yeast extract *(14)*. Other important aspect refers to the fact that the strain *S. cerevisiae* W303-181 belongs to a

public Institution (University of São Paulo), eliminating the payment of royalties to a foreign supplier by a Brazilian company, if the process is scaled up to an industrial plant.

Conclusions

The data presented lead to conclude that the fed-batch process was suitable for G6PD production insofar as the linear or exponential decreasing feeding strategy was used. Moreover, *S. cerevisiae* W303-181 had a behavior like any yeast of the genus *Saccharomyces* in which growth and G6PD synthesis are coupled events. The G6PD synthesis depended on the amount of glucose and micronutrients present in the medium culture. The cell free extract achieved had a G6PD activity (350 U/L) comparable with those G6PD preparations already marketed.

Acknowledgments

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