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Use of Glucose Oxidase in a Membrane Reactor for Gluconic Acid Production

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Abstract

This article aims at the evaluation of the catalytic performance of glucose oxidase (GO) (EC.1.1.3.4) for the glucose/gluconic acid conversion in the ultrafiltration cell type membrane reactor (MB-CSTR). The reactor was coupled with a Millipore ultrafiltration-membrane (cutoff of 100 kDa) and operated for 24 h under agitation of 100 rpm, pH 5.5, and 30°C. The experimental conditions varied were the glucose concentration (2.5, 5.0, 10.0, 20.0, and 40.0 m*M*), the feeding rate (0.5, 1.0, 3.0, and 6.0/h), dissolved oxygen (8.0 and 16.0 mg/L), GO concentration (2.5, 5.0, 10.0, and 20.0 U_{GO}/mL), and the glucose oxidase/catalase activity ratio (U_{GO}/U_{CAT}) (1 : 0, 1 : 10, 1 : 20, and 1 : 30). A conversion yield of 80% and specific reaction rate of 40×10^{-4} mmol/h · U_{GO} were attained when the process was carried out under the following conditions: D = 3.0/h, dissolved oxygen = 16.0 mg/L, [*G*] = 40 m*M*, and (U_{GO}/U_{CAT}) = 1 : 20. A simplified model for explaining the inhibition of GO activity by hydrogen peroxide, formed during the glucose/gluconic acid conversion, was presented.

Index Entries: Catalase; gluconic acid; glucose oxidase; membrane reactor; glucose; continuous process.

Introduction

The oxidation of glucose into gluconic acid (GA) is one of great interest, not only owing to the high availability of glucose (attained from sucrose and starch, the main and abundant natural sources) but also to the high market demand for GA—over 10,000 t/yr worldwide *(1)*,—a product largely used in the food (as acidulant and surface bleaching), chemical (as surface cleansing agent), and pharmaceutical (gluconate salts) industries *(2)*. GA is obtained from the oxidation of glucose either through the chemical catalysis (bismuth, palladium, platinum, or gold immobilized into active charcoal used as catalyst) or by microbial conversion *(3,4)*. Fermentation is the main process for GA production in which strains of *Aspergillus niger*, *Gluconobacter suboxydans*, or *Acetobacter methanolicus* are used *(4)*. The preference for the

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biotechnological process is mainly owing to the good GA yield attained (>80%), the small amount of byproducts formed, the utilization of commercial-grade glucose, and the generation of environmentally inoffensive residues. Despite these advantages on the microbial process, intrinsic problems to be addressed include subsequent cell separation, as well as the discharge and destination of huge volumes of liquid after GA separation. However, the use of glucose oxidase (GO) as catalyst is another valuable way to perform the G/GA conversion. The enzyme catalysis, which presents all the advantages cited for the fermentation plus the mild operational conditions, the low energy requirement, the high specificity for glucose, the generation of nonpollutant residues, and the possibility of carrying out the conversion in a continuous reactor.

Glucose oxidase (EC.1.1.3.4) is a well-characterized enzyme (molecular mass: 150–190 kDa; has two glycoprotein chains linked by disulfide bonds, with each chain having one ferrous ion and one FAD prosthetic group) and largely used in analytical techniques and industrial processes *(5–7)*. Because of the presence of the Fe^{2+}/FAD system in the GO structure, the enzyme is sensitive to the presence of any redox agent, mainly hydrogen peroxide, which is one of the reaction products and a strong redox substance. According to Tomotani et al. *(8)*, H_2O_2 is a reversible noncompetitive inhibitor of GO, whose inhibition constant is equal to 1.22 m*M*.

Throughout the article several continuous processes—using packed-bed, fluidized-bed, or membrane reactorfor the G/GA conversion catalyzed by GO are described *(7,8)*. Over the last 10 yr the membrane reactor has received high attention for the production of a variety of products *(8)* mainly because of the possibility of using the catalyst in soluble or insoluble form. Such an option is not easily available for the other types of continuous reactors.

A membrane reactor can be assembled through two distinct designs, i.e., as continuous stirred tank reactor (CSTR) coupled with a semipermeable membrane (MB-CSTR) or as a hollow-fiber reactor (a tank without stirring filled with a sheaf of straight-lined hollow-fibers tubes of semipermeable membrane). The MB-CSTR is shaped by connecting in series the CSTR and the 100 kDa UF-membrane module (MB-$CSTR_{Se}$) or by adapting the UF-membrane to the bottom of the CSTR as in a stirring ultrafiltration cell (MB-$CSTR_{UFC}$). The membrane/catalyst arrangement can or cannot involve an interaction between them. If they are linked, the membrane acts as catalysis and separation surface simultaneously; otherwise, it functions only as a separation surface. When the enzyme is in the soluble form, the MB-$CSTR_{Se}$ requires recycling of the catalyst, whereas the MB-$CSTR_{UFC}$ does not. Moreover, the enzyme used may be immobilized in nonmembranous materials (for instance, ion exchange resin beads) *(9)*, in case which the MB-$CSTR_{UFC}$ is preferred over MB-$CSTR_{Se}$ because no recycling of the reaction medium is required. An additional advantage of the MB-$CSTR_{UFC}$ over MB-$CSTR_{Se}$ is the elimination of the pumping step,

surely leading to the reduction of the overall costs, mainly those related to the energy consumption and maintenance of the pumping system.

According to the literature, the enzymatic oxidation of glucose is preferentially conducted in MB-$CSTR_{Se}$ in which the GO is united to the membrane. Nonetheless, there is little information on MB-$CSTR_{UFC}$ using soluble GO, in spite of presenting operational characteristics of homogeneous catalysis, high activity per unit of volume, and absence of conformational and diffusional effects. If needed, the membrane reactor also allows operation under aseptic conditions as well as with multienzymatic systems *(8)*.

In previous work *(8)*, it was reported that the MB-$CSTR_{UFC}$ was suitable for the G/GA conversion provided that the H_2O_2 inhibition was minimized. Moreover, a conversion yield of about 75% was achieved under the following conditions: pH 5.5, 30°C, *D* (feeding rate) = 0.15/ min, agitation of 100 rpm, 2.5 m*M* glucose, 1.0 mg/mL of GO, and dissolved oxygen (DO) of 7.0 mg/L. However, the low substrate concentration and the short residence time used in the process, though adequate for controlling the hydrogen peroxide formation, are inadequate for a future scaling-up.

This work aims at the evaluation of the catalytic performance of GO for the G/GA conversion in the MB-$CSTR_{UFC}$. The experimental conditions that varied were the glucose concentration (2.5, 5.0, 10.0, 20.0, and 40 m*M*), the feeding rate (0.5, 1.0, 3.0, and 6.0/h), DO (8.0 and 16.0 mg/L), and the GO/catalase activity ratio (U_{GO}/U_{CAT}) (1 : 0, 1 : 10, 1 : 20, and 1 : 30).

Materials and Methods

Chemicals

GO from *A. niger* and bovine catalase were purchased from Sigma (St. Louis, MO). One gram of GO corresponds to 5100 units. One unit (U_{GO}) will oxidize 1 μmol of β-D-glucose to GA and H_2O_2 per min at 35°C and pH 5.1. One milligram of catalase corresponds to 2350 units. One unit (U_{CAT}) will decompose 1 μmol of H_2O_2 per min at 25°C and pH 7.0, while the H_2O_2 concentration falls from 10.3 to 9.2 m*M*. The 100-kDa UF-membrane (PLHK07610, made of regenerated cellulose) was purchased from Millipore (Bedford, MA). All other chemicals were of analytical grade.

Membrane Reactor

A 10-mL MB-$CSTR_{UFC}$ (Bioengineering AG, Wald, Germany) was used in all tests. The reactor is a 316-L stainless steel cylinder, whose bottom has an inlet and an outlet for the external water bath for temperature control. The diameter of the UF-membrane used was 63 mm. The reactor can be sterilized (autoclave up to 134°C for 30 min) and resists high temperatures (up to 150°C) and corrosion by most substances (except strong acids, pH < 1.0; and alkalis, pH > 12.0). Moreover, it has a safety valve (set to nominal six bar pressure limit) and can be coupled to a dosing pump, pressure probe, sterile filter, and bubble trap.

Membrane Reactor Tests

Ten milliliters of buffered GO solution (2.5, 5.0, 10.0, or 20.0 U_{GO}/mL in 0.01*M* acetic acid/acetate buffer, pH 5.5) was poured inside the MB-$CSTR_{UFC}$, which had a UF-membrane (PLHK07610) with a molecular mass cutoff of 100 kDa. The reactor was fed continuously with 2.5, 5.0, 10.0, 20.0, or 40.0 m*M* glucose buffered solution (0.01 *M* acetic acid/acetate buffer, pH 5.5) at a feeding rate of 0.5, 1.0, 2.0, 3.0, or 6.0/h. The reaction was carried out for 24 h at 30°C and an agitation of 100 rpm. Pure oxygen was bubbled into the glucose solution, so that the DO concentration in the inlet solution remained around 8.0 mg/L or 16.0 mg/L. Aliquots taken from the outlet solution were measured for the concentration of glucose and H_2O_2. The yield (*Y*) and the specific reaction rate (*r*) were calculated through the Eqs. 1 and 2, respectively. The conditions used in all tests realized were presented in Table 1. Each test was carried out in duplicate. When a difference over 5% on *Y* and *r* was observed, then the test was repeated two or more times.

$$Y\ (\%) = \frac{G_{cons}}{G_0} \times 100 \tag{1}$$

$$r\ (\text{mmol/h} \times U_{GO}) = \frac{Q \times G_{cons}}{1000 \times U_{GO}} \tag{2}$$

where $[G]_0$ is the inlet glucose concentration, $[G]_{cons}$ is the $[G]_0 - [G]_{inlet}$, Q is the volumetric rate (mL/h), and U_{GO} is the GO units used. As the stoichiometry of G/GA conversion catalyzed by GO is 1 mol of glucose generating 1 mol of GA and 1 mol of H_2O_2 *(7)*, then the $[G]_{cons}$ is equal to the concentration of hydrogen peroxide formed.

Analytical Techniques

Determination of Glucose

The concentration of glucose was measured by using an enzymatic peroxidase/GO kit (Laborlab, São Paulo, SP, Brazil). The procedure was accomplished by mixing 10 μL of the sample with 1.0 mL of peroxidase/GO solution, and incubated at 37°C for 10 min. After that, the absorbance was read in a spectrometer (Beckman DU 640; Beckman Coulter, Fullerton, CA) at λ = 500 nm. The standard curve was attained using glucose solutions whose concentration range was between 0.04 and 0.20 mg/mL. The regression curve established was

$$ABS_{500nm} = 0.168 \times [G] + 0.152\ (r = 0.9990) \tag{3}$$

where [*G*] is the glucose concentration (mg/mL).

The standard deviation and coefficient of variation related to this method were 0.010 mg/mL and 4.5%, respectively.

Table 1
Conditions Under Which all Continuous Experiments Were Conducted and Respective Average Conversion (Y) and Specific Reaction Rate (r) Attained[a,b]

Test (n)	[G] (mM)	[GO] (U_{GO}/mL)	[CAT] (U_{CAT}/mL)	D (h^{-1})	DO (mg/L)	R (mmol/ $h \cdot U_{GO}$) × 10^4	Y (%)
1	2.5	10	–	1.0	8.0	1.9	48
2	2.5	10	–	3.0	8.0	2.7	29
3	2.5	10	–	6.0	8.0	1.9	43
4	2.5	10	–	1.0	16.0	2.3	94
5	2.5	10	–	3.0	16.0	3.7	50
6	2.5	10	–	6.0	16.0	3.1	19
7	5.0	2.5	–	3.0	16.0	24	41
8	5.0	5	–	3.0	16.0	11	37
9	5.0	10	–	3.0	16.0	7.1	48
10	5.0	20	–	3.0	16.0	5.1	70
11	5.0	2.5	–	1.0	16.0	9.1	45
12	5.0	5	–	1.0	16.0	4.7	48
13	5.0	5	–	2.0	16.0	9.5	48
14	5.0	10	–	0.5	16.0	3.4	83
15	5.0	10	–	1.0	16.0	1.9	39
16	5.0	20	–	1.0	16.0	2.9	88
17	5.0	10	100	3.0	16.0	13	88
18	5.0	10	200	3.0	16.0	13	88
19	5.0	10	300	3.0	16.0	13	88
20	10	10	200	3.0	16.0	25	85
21	20	10	200	3.0	16.0	40	80
22	40	10	200	3.0	16.0	41	35

[a]In all tests the pH, temperature, and agitation were maintained at 5.5, 30°C, and 100 rpm, respectively.
[b]The yield (Y) and the specific reaction rate (r) values presented are an average of all the time points.

Determination of Hydrogen Peroxide

H_2O_2 concentration was determined through the ultraviolet absorption method ($\lambda = 240$ nm) as described by Bergmeyer *(10)*. A standard curve was established by measuring the absorbance of H_2O_2 solution, whose concentration ranged from 0.18 to 18 mM. A pharmaceutical grade H_2O_2 was used. The regression curve established was

$$ABS_{240nm} = 4.12 \times 10^{-2} [H_2O_2] - 6.97 \times 10^{-4} \quad (r = 0.9995) \qquad (4)$$

where $[H_2O_2]$ is the H_2O_2 concentration (mM).The standard deviation and coefficient of variation related to this method were 0.86 mM and 0.33%, respectively.

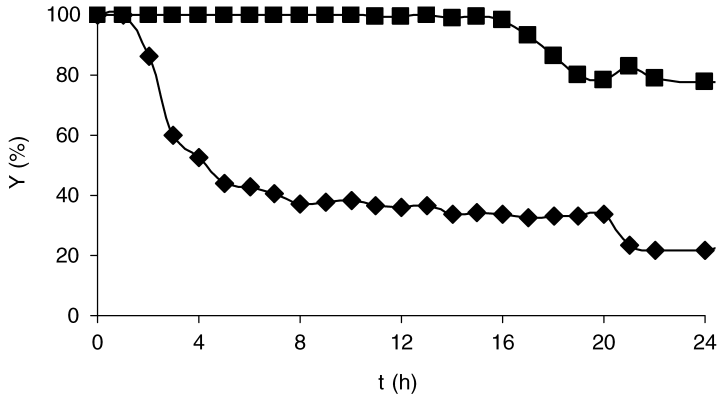

Fig. 1. Variation in conversion during continuous glucose oxidation catalyzed by GO for test 1 (◆) and test 4 (■).

Determination of DO

DO was measured using an oximeter (Digimed, model DM40, São Paulo, Brazil). In all experiments the aqueous glucose solution was bubbled with pure oxygen for attaining the desired concentration 8.0 or 16.0 mg/L.

Results and Discussion

Taking into account tests 1–6 in which only DO and *D* were varied, we observed that tests 1 (D = 1.0/h and DO = 8.0 mg/L) and 4 (D = 1.0/h and DO = 16.0 mg/L) presented the highest G/GA conversion yields, respectively, 48% and 94% (Table 1). The G/GA conversion yield of 94% achieved in test 4 was far better than that found by Tomotani et al. *(8)* pointing the importance of the amount of DO in the reaction medium for the GO catalysis. According to Bright *(11)* the G/GA conversion by GO is a two steps process (First: $GO\text{-}FAD_{ox} + G \rightarrow GO\text{-}FAD_{red}$ + glucone-δ-lactone; Second: $GO\text{-}FAD_{red} + O_2 \rightarrow H_2O_2 + GO\text{-}FAD_{ox}$) in which the first one is the limiting step. Thereby, under unsaturated O_2 concentration the glucose might inhibit the GO.

Figure 1 shows that a steady state of 16 h occurred in tests 4 (average Y near 100% from t = 0 h to t = 16 h) and 1 (average Y near 40% from t = 4 h to t = 20 h). Moreover, the G/GA conversion yield diminished in tests 4 (Y varied from 100 to 80%) and 1 (Y varied from 40 to 25%), respectively, at intervals of 16–24 h and 20–24 h of continuous processing. The perturbation of the stationary phase after several residence times might be owing to the accumulation in the reaction medium of GO molecules containing the iron atom in the Fe^{+3} form instead of Fe^{+2} (the most adequate form for full GO catalysis). In some extension, the redox state of the iron atom contributes to the loss in enzyme stability. According to Tomotani et al. *(8)*, a fraction of $GO\text{-}Fe^{+3}$ molecules could arise owing to the accumulation of hydrogen

peroxide in the reaction medium, because the potential of reduction of the pair H_2O_2/H_2O (+1.77V) is higher than the pair Fe^{+3}/Fe^{+2} (+0.771V).

The G/GA conversion yield and the specific reaction rate varied according to the feeding rate or the residence time (inversely correlated with *D*) used. The highest *Y* (94%) and *r* (3.7×10^{-4} mmol/h $\cdot U_{GO}$) occurred at $D = 1.0$/h (test 4) and $D = 3.0$/h (test 5), respectively (Table 1). Thereby, the period under which all reacting species (GO, G, O_2, and H_2O_2) are left in contact inside the reactor might be adequately set, in order to balance the two steps reaction for the G/GA conversion, as referred earlier.

Undoubtedly, the G/GA conversion yield attained here was significantly improved as compared with that described by Tomotani et al. *(8)*. However, when a scale-up of this process is envisaged, other aspects such as GO and glucose concentration and decomposition of the hydrogen peroxide accumulated in the reaction medium must be evaluated.

The concentration of glucose solution fed in tests 7–10 was set at 5 m*M* and the GO concentration varied from 2.5 to 20 U_{GO}/mL (Table 1). As the GO concentration was increased, the specific reaction rate (*r*) decreased from 24×10^{-4} mmol/h $\cdot U_{GO}$ (test 7) to 5.1×10^{-4} mmol/h $\cdot U_{GO}$ (test 10), whereas the conversion yield increased from 41% (test 7) to 70% (test 10). By varying the feeding rate at fixed amounts of glucose and GO, as in the tests 11–16 (Table 1), we conclude that *D* is not an important parameter for achieving high *Y* and *r* simultaneously. Probably, the presence of hydrogen peroxide in the reaction medium has a role on the final values achieved for *Y* and *r* because it interferes with the overall GO redox state, as mentioned previously. Such influence is easily understood if the H_2O_2 strong redox capability is considered. To circumvent the undesirable effect of hydrogen peroxide on the G/GA conversion, the addition of catalase (EC.1.11.1.6) in the reaction medium was evaluated (tests 17–19).

The use of catalase clearly affected the conversion yield and the specific reaction rate as can be seen through the comparison of tests 9 and 17–19 (Table 1). The specific reaction rate (*r*) and the yield (*Y*) increased simultaneously about 46% regarding the test carried out without catalase (test 9). At glucose concentration of 5 m*M* the ratio GO/catalase (U_{GO}/U_{CAT}) (test 17: [1 : 10]; test 18: [1 : 20]; and test 19: [1 : 30]) did not affect the reaction rate and the yield (Table 1). In presence of catalase the hydrogen peroxide concentration in the medium diminishes markedly (Fig. 2) leading to the more predictable situation in which *Y* and *r* increase or decrease at the same time. Moreover, the steady-state condition is maintained along the whole process (Fig. 2), differently from what occurred in test 4, whose steady-state condition remained till $t = 16$ h of continuous process (Fig. 1). This result corroborates, albeit indirectly, the kinetic model for the action of GO in presence of H_2O_2 proposed by Bao et al. *(12)*, which assumes a competitive inhibition pattern by H_2O_2 with respect to the O_2 for the GO reduced form ($GO\text{-}FAD_{red}$). Another possibility would be the action of the hydrogen peroxide on the redox state of the iron atom instead

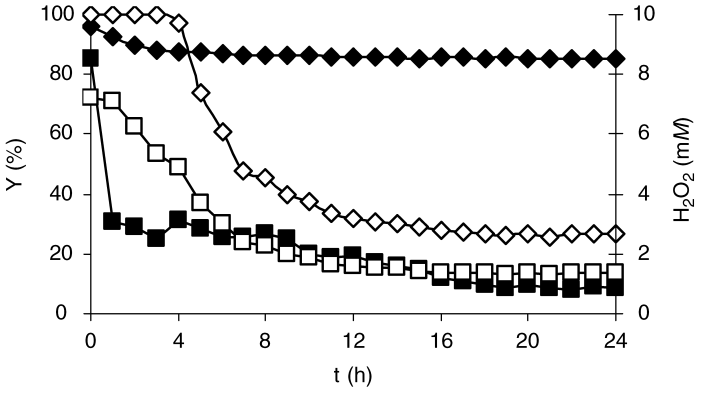

Fig. 2. Variation in conversion during continuous glucose oxidation catalyzed by GO for test 9 (◇) and test 17 (◆). Variation of the concentration of hydrogen peroxide in the effluent during the process for test 9 (□) and test 17 (■).

of on the FAD redox state *(8)*. Introducing these assumptions in the Bright's model *(10)*, it becomes a four steps process, i.e.,

$$\text{First:} \quad \text{GO-FAD}_{ox} + \text{G} \rightarrow \text{GO-FAD}_{red} + \text{glucone-}\delta\text{-lactone}$$

$$\text{Second:} \quad \text{GO-FAD}_{red} + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{GO-FAD}_{ox}$$

$$\text{Third:} \quad \text{GO-FAD}_{red} + \text{H}_2\text{O}_2 \rightarrow \text{GO-FAD}_{red} + \text{H}_2\text{O}_2$$

$$\text{Fourth:} \quad \text{GO-Fe}^{+2} + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \frac{1}{2}\,\text{O}_2 + \text{GO-Fe}^{+3}$$

Therefore, the addition of catalase promotes the H_2O_2 decomposition—as consequence, the third and fourth steps are eliminated—leaving the $GO\text{-}FAD_{red}$ available to be oxidized by the oxygen, and the GO turnover is completed.

As the role of catalase in circumventing the inhibition caused by H_2O_2 on the GO activity was remarkable, and knowing that the H_2O_2 generation is stoichiometrically related to glucose oxidation, the study of G/GA conversion using more concentrated glucose solutions was explored. Tests 20–22 were carried out with glucose solutions of 10, 20, and 40 m*M*, and the results presented in Fig. 3 and Table 1.

Test 21, carried out with 20 m*M* glucose solution, presented the highest r (40×10^{-4} mmol/h · U_{GO}) and Y (80%), whereas test 22, carried out with 40 m*M* glucose solution, presented a high r (41×10^{-4} mmol/h · U_{GO}) but low Y (35%) (Table 1). On one hand, the similar specific reaction rates observed indicate that catalase at concentration of 200 U_{CAT}/mL was enough for maintaining the hydrogen peroxide concentration at noninhibitory level (Fig. 3). On the other hand, the GO at concentration of 10 U_{GO}/mL was insufficient to cope with 40 m*M* glucose solution, as shown by the sharp reduction of the G/GA conversion yield (test 22). Therefore, for each glucose concentration over 20 m*M* an adequate U_{GO}/U_{CAT} ratio might be adjusted.

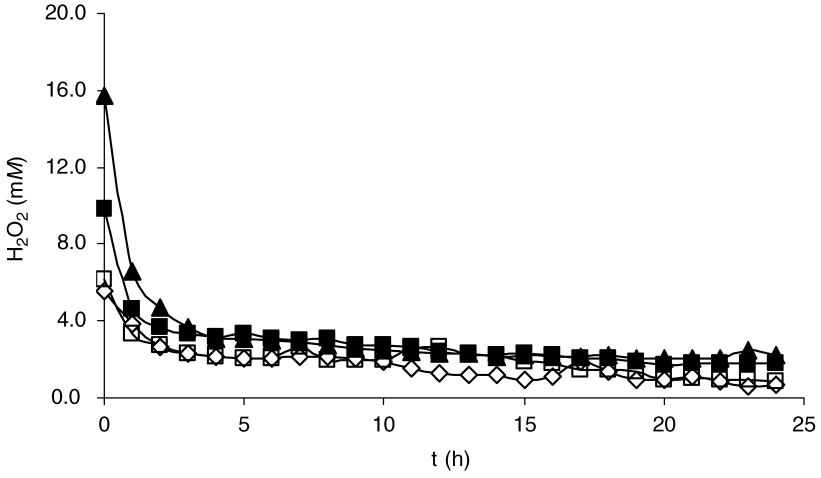

Fig. 3. Variation of the concentration of hydrogen peroxide in the effluent during the continuous process for tests (◇) 18, (□) 20, (▲) 21, and (■) 22.

In the present work, tests with still more concentrated glucose solutions were not made because the high bubbling caused by the oxygen generated from H_2O_2 decomposition augmented the 10 mL-MB-$CSTR_{UFC}$ internal pressure nearing the up limit of six bar. Going over that pressure would be quite dangerous. Certainly, this handicap might not occur or might be minimized at least, if a large UF-membrane reactor was used. Moreover, such "*in situ*" oxygen generation would allow the elimination of the previous saturation of the substrate solution, which should simplify the overall process.

Finally, comparing the results obtained here with those found by Tomotani et al. *(8)* the boundary for the glucose concentration, which could be used in the G/GA conversion, was enlarged eightfold, i.e., up to 20 m*M*.

Conclusions

The data presented led us to conclude that the reaction medium must be saturated with oxygen (16.0 mg/L), in order to avoid the GO inhibition by glucose (its natural substrate). However, the hydrogen peroxide—a byproduct resulting from the G/GA conversion—can hinder the GO catalysis either through binding to the GO-FAD_{red} (an intermediate form of the enzyme) or by oxidizing the Fe^{+2} to Fe^{+3} (another cofactor present in the GO molecule). Such effects are circumvented by maintaining the hydrogen peroxide concentration below 2 m*M* along the whole process, which is achieved by adding catalase into the reactor. The ratio GO/catalase must be adjusted for each glucose concentration over 2.5 m*M*. Finally, the advance of this research might be based on the best conditions for the G/GA conversion established here, i.e., [*G*] = 20 m*M*, [*GO*]/[*CAT*] = 1 : 20, *D* = 3.0/h, 30°C, DO = 16.0 mg/L, and pH 5.5.

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