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Multienzymatic Sucrose Conversion into Fructose and Gluconic Acid through Fed-Batch and Membrane-Continuous Processes

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Abstract Multienzymatic conversion of sucrose into fructose and gluconic acid was studied through fed-batch and continuous (in a membrane reactor) processes. The law of substrate addition (sucrose or glucose) for the fed-batch process which led to a yield superior to 80% was the decreasing linear type, whose feeding rate (ϕ; L/h) was calculated through the equation: $\phi = \phi_o - k.t$, where ϕ_o (initial feeding rate, L/*h*), *k* (linear addition constant, L/h^2), and *t* (reaction time, *h*). In the continuous process, the yield of conversion of sucrose (*Y*) was superior to 70% under the following conditions: dilution rate=0.33 h^{-1}, total duration of 15 h, pH 5.0, 37 °C and initial sucrose concentration of 64 g/L (*Y*=92%), 100 g/L (*Y*=83%), or 150 g/L (*Y*=76%).

Keywords Invertase · Glucose oxidase · Catalase · Gluconic acid · Membrane reactor

Introduction

Biotechnology processes catalyzed by enzymes have some advantages as compared with chemical processes, i.e., they are carried out under smooth pH and low temperature, which facilitate the handling of sensitive substances, they present high stereochemical selectivity and they do not generate unfriendly byproducts to the environment [1]. It must be pointed out that the simultaneous use of two or more enzymes—a successful strategy for biosensors [2], enzymatic kits for clinical diagnosis [3], and the continuous coenzyme regeneration concomitantly to the coupled action of dehydrogenases [4]—allows to convert sequentially substrates present into the medium, even those generated along the reaction as intermediates.

Enzymatic catalysis has generally been used in reactions based on the transformation of one substrate into one product on molar basis [5]. However, multienzyme systems could be used as catalysts for converting one substrate into two or more products through processes

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involving two or more sequence of chemical reactions. In this case, the overall conversion would be carried out in a one-step process [6]. Although, the multienzyme system is successfully employed in analytical protocols, its use in large-scale processes is rarely described. As a seldom example, Sheu et al. [7] described the conversion of sucrose into fructooligosaccharides and gluconic acid in a one-step process by using invertase, glucose oxidase, and catalase, simultaneously.

In previous works related to the conversion of sucrose into glucose and fructose by using soluble or immobilized invertase [8] and to the oxidation of glucose to gluconic acid and hydrogen peroxide by glucose oxidase [9, 10], it was envisaged the possibility for converting the sucrose into fructose and gluconic acid by a one-step process, in which those enzymes would be used simultaneously. Besides, Tomotani et al. [11] showed that the hydrogen peroxide, a by-product of the glucose oxidation, should be removed from the reaction medium, suggesting the use of catalase as an auxiliary enzyme. Thereby, the perspective of using invertase, catalase, and glucose oxidase simultaneously deserved to be studied in laboratory scale.

As the hydrogen peroxide over 1.3 mM inhibits the action of the glucose oxidase [11] so the conversion [sucrose/(fructose + gluconic acid)] must be carried out through processes that allow controlling the amount of hydrogen peroxide present into the reaction medium. Among all types of available processes, the fed-batch and the continuous processes are the most appropriate, because they allow maintaining the hydrogen peroxide concentration below 1.3 mM through the controlled addition of sucrose into the reactor [9, 12]. Therefore, in the fed-batch type process (in which the substrate is added step-by-step according to an addition law) the hydrogen peroxide is decomposed by catalase as soon as it is produced, whereas in the continuous type (in which the substrate is introduced through a fixed feeding rate) the hydrogen peroxide decomposition occurs simultaneously to its continuous removal from the reaction medium.

Among the continuous bioreactors, the fluidized-bed ones are the most suitable for operating multienzyme systems, because problems like mass transfer and establishment of pH and temperature gradients in the reaction medium are negligible [13]. In particular, the so-called unimodular membrane reactor—a continuous stirred tank reactor (CSTR) coupled with an ultrafiltration membrane, which is set at the bottom of the vessel—is a kind of fluidized bed reactor which can easily operate with both soluble or insoluble enzymes [9, 12].

In this work, the conversion of sucrose to fructose and gluconic acid by the simultaneous action of invertase, glucose oxidase, and catalase was evaluated through the fed-batch (the substrate solution was added to the reactor according to the following adding laws: constant, linear increasing, linear decreasing, exponential increasing, and exponential decreasing) and the membrane-continuous processes.

Material and Methods

Material

Invertase (EC.3.2.1.26) from *Saccharomyces cerevisiae* was purchased from Merck® (1 mL of invertase presents 1,765 U, where 1 U=1 mg total reducing sugars (TRS)/min, measured at pH 5.0 and at 37 °C; TRS; Km=40.3 mM [14]); glucose oxidase (EC.1.1.3.4) from *Aspergillus niger* (from SIGMA®—1 solid g has 5,000 U, where 1 U=1 μmol of H_2O_2 formed/min, measured at pH 5.0 and at 37 °C; Km=29 mM [11]) and catalase (EC

1.11.1.6) from bovine liver (SIGMA®—1 solid mg has 2,950 U, where 1 U=1 μmol of H_2O_2 consumed/min, measured at pH 4.6 and 37 °C). The continuous tests were made in CSTR (unimodular-MB, Millipore® ultrafiltration cell) and with a regenerated cellulose membrane with a molecular weight cut-off (MWCO) of 100 kDa (Millipore®). All of the other chemicals were of analytical grade. The hydrogen peroxide was of pharmaceutical grade preparation (10 V).

Methods

Enzymatic Assays

Invertase In a 250-mL beaker, 108 mL of 0.01 M acetate buffer (pH 3.5; 4.0; 4.5; 5.0; 5.5 or 6.0) was added. The beaker was introduced into a water bath at 37 °C. After 10 min, an amount of sucrose was dissolved under agitation (100 rpm) in order to reach an initial concentration of 100 g/L. After 5 min, 12 mL of the invertase solution (3.43 U) was added into the beaker. After that, a chronometer was started. Aliquots of 0.5 mL were taken every minute until completing the total reaction time of 6 min. The concentration of TRS in each sample was determined by using the Somogyi–Nelson's method [15]. Before adding the enzyme, a sample of 0.5 mL of the sucrose solution was taken to measure the initial TRS (t=0).

The enzymatic activity (v_I) was calculated by plotting the graph of TRS (mg) versus time (min). One unit of invertase (U_I) was defined as the amount of TRS in milligrams formed per minute and per milliliter of reaction medium under the test conditions (pH 5.0 and 37 °C).

The stability of the invertase at pH 5.0 and 37 °C was evaluated by preparing six tubes containing 12 mL of invertase solution (3.43 U, acetate buffer 0.01 M and pH 5.0). They were left in a water bath at 37 °C for 0, 0.5, 1, 10, 20, and 30 h, after which time the residual enzymatic activity was determined.

Glucose Oxidase

In a 250-mL beaker, 125 mL of 0.01 M acetate buffer (pH 4.0, 4.5, 5.0, 5.5, 6.0, or 6.5) was added. The beaker was introduced into a water bath at 37 °C. During the entire process, air was bubbled into the reaction medium in order to keep it saturated with oxygen (4 mg/L). An aliquot sample of 1 mL was removed as a blank. After 10 min, an amount of glucose was dissolved under agitation (100 rpm) in order to reach an initial concentration of 4.5 g/L. A sample of 1 mL of the glucose solution was taken to measure the initial concentration of H_2O_2 (t=0). Afterwards, 5 min later, 25 mL of the glucose oxidase (GO) solution (180 U) was added into the beaker and a chronometer was started. Aliquot samples of 1 mL were removed every 10 min until completing the total reaction time of 60 min. Each sample was poured into a boiling water bath in order to stop the reaction. The measurement of H_2O_2 formed during the reaction was made by reading the absorbance at 240 nm. The GO activity (v_{GO}) was calculated by plotting H_2O_2 (mg) versus time (min). One unit of glucose oxidase (U_{GO}) was defined as the amount of H_2O_2 in milligrams formed per minute and per milliliter of reaction medium under the test conditions (pH 5.0 and 37 °C).

The stability of the GO at pH 5.0 and at 37 °C was evaluated by preparing six tubes containing GO (180 U) dissolved in 25 mL of 0.01 M acetate buffer (pH 5.0). They were left in a water bath at 37 °C for 0, 0.5, 1 h, 10, 20, and 30 h, after which the residual enzymatic activity was determined.

Catalase

A volume of 30 mL of hydrogen peroxide solution, containing in overall mass basis 30, 60, 120, 240, 450, or 900 mg of H_2O_2, was mixed in a 250-mL beaker with 96 mL of 0.01 M acetate buffer (pH 5.0), being the mixture immersed into a water bath at 37 °C. The reaction, carried out for 30 min, was started by adding 24 mL of catalase solution (1,230 U/mL). Aliquots of 1 mL were taken at each 5 min, put into Eppendorf tubes, which were immersed into a boiling water bath for 5 min, in order to inactivate the enzyme. After cooling, the solution was introduced into a 1-mL cuvette (1 cm light path) and the residual hydrogen peroxide was determined by reading the absorbance of the solution at 240 nm. The catalase activity (v_{cat}) was calculated by plotting H_2O_2 (mg/mL) versus time (min). One unit of catalase activity (U_{cat}) was defined as the amount of H_2O_2 consumed per min and per milliliter of reaction medium at pH 5.0 at 37 °C. The interval from 0 to 5 min of reaction was set for evaluating the consumption of hydrogen peroxide.

Catalase activity was calculated through the equation:

$$v_{cat} = \left([H_2O_2]_i - [H_2O_2]_{5min}\right) \div 5 \quad (1)$$

where, $[H_2O_2]_i$=Hydrogen peroxide concentration at t =0 and $[H_2O_2]_{5min}$=residual hydrogen peroxide concentration at 5 min of reaction.

Tests were also conducted in which both catalase and hydrogen peroxide were added according to a constant fed-batch mode. Thereby, the reaction was started by adding volumes of 6 and 4 mL taken, respectively, from a 30 mL H_2O_2 solution (75 g/L) and 24 mL catalase solution (2,500 U/mL). Then, the same volumes were added at each 5 min up to 25 min of reaction. Aliquots of 1 mL were taken immediately before the addition of the catalase and hydrogen peroxide solutions. After this, all other conditions were the same as described above.

A blank test was carried out, as described above, without addition of hydrogen peroxide. This blank aimed to discount the light absorption at 240 nm by the catalase present into the medium [16].

The stability of the catalase at pH 5.0 and at 37 °C was evaluated by preparing 11 tubes containing catalase (29.520 U) dissolved in 24 mL of 0.01 M acetate buffer (pH 5.0). They were left in a water bath at 37 °C up to 30 h, being the residual activity measured at each 3 h as described above.

Sucrose Hydrolysis (Batch Process)

In a 500-mL beaker, 250 mL of 0.01 M acetate buffer (pH 5.0) was added, which was immersed into a water bath at 37 °C. After 10 min, 19.2 g of sucrose was dissolved under an agitation of 100 rpm followed by addition of 50 mL of an aqueous solution of invertase (776.6 U, pH 5.0). Samples were removed every 10 min from the reaction medium. The total reaction time was 1 h. The TRS was measured through the Somogyi's method.

Fed-Batch Process (Sucrose Hydrolysis and Glucose Oxidation)

Table 1 shows the substrate addition laws (sucrose or glucose), aiming to optimize sucrose hydrolysis by invertase and glucose oxidation by GO.

Table 1 The substrate addition laws used in fed-batch tests

Type of adittion	Equations	Integrated form
Constant feeding	$\phi = (V_f - V_o)/t$	$\phi = (V_f - V_o)/t_f$
Linear increasing	$\phi = \phi_o + k.t$	$(V - V_o) = \phi_o.t + k.t^2/2$
Linear decreasing	$\phi = \phi_o - k.t$	$(V - V_o) = \phi_o.t - k.t^2/2$
Exponential increasing	$\phi = \phi_o.e^{k't}$	$(V - V_o) = \phi_o/k'.(e^{k't} - 1)$
Exponential decreasing	$\phi = \phi_o.e^{-k't}$	$(V - V_o) = \phi_o/k'.(e^{-k't} - 1)$

The symbols of the equations presented are ϕ (L/h, feeding rate), ϕ_o (L/h, initial feeding rate), k (L/h^{2,} linear addition constant), k' (1/h, exponential addition constant), t (h, addition time), V_o (L, initial reaction volume), V_f (L, final reaction volume), V (L, volume inside the reactor at any time of reaction)

Sucrose Hydrolysis

In a 500-mL beaker, 50 mL of 0.05 M acetate buffer (pH 5.0) and 50 mL of an enzyme solution (pH 5.0, 776.6 U) were added. The reaction volume was increased from 100 up to 400 mL for 1 h by adding volumes of sucrose solution (64 g/L), which were calculated through the integrated equations shown in Table 1.The reaction was made at 37 °C and under an agitation of 100 rpm. Every 6 min and before each sucrose addition, samples were removed from the reaction medium for analytical purposes.

Glucose Oxidation

In a 500-mL beaker, 50 mL of 0.05 M acetate buffer (pH 5.0) and 50 mL of GO solution (7,000 U) were added. The reaction volume was increased from 100 up to 400 mL for 4 h by adding volumes of glucose solution (32 g/L) containing catalase (236,000 U), which were calculated through the integrated equations shown in Table 1.The reaction was carried out under a constant agitation (100 rpm) and at 37 °C. During the entire process, air was bubbled into the reaction medium in order to keep it saturated with oxygen (4 mg/L). Samples were removed from the reaction medium every 24 min and before the addition of the glucose–catalase solution for analytical purposes.

Multienzymatic Conversion of Sucrose into Fructose and Gluconic Acid (Fed-Batch Process)

In a 500-mL beaker, 50 mL of buffered invertase solution (pH 5.0, 776.6 U) and 50 mL of buffered GO solution (pH 5.0, 7,000 U) were added. The reaction volume was augmented from 100 up to 400 mL for 4 h by adding volumes of buffered sucrose solution (64 g/L, pH 5.0) containing catalase (236,000 U). The volumes were calculated through the equation: $(V - V_o) = \phi_o.t - k.t^2/2$ (Table 1). The reaction was carried out at 37 °C under agitation of 100 rpm. During the entire process, air was bubbled into the reaction medium in order to keep it saturated with oxygen (4 mg/L). For analytical purposes, samples were removed from the reaction medium every 24 min and before the addition of the sucrose–catalase solution.

The sequence for the multienzyme conversion of sucrose (S) to fructose (F), gluconic acid (GA), and remaining glucose (G) and H_2O_2 can schematically be envisaged through the equations:

$$S \xrightarrow{(\textbf{invertase})} F + G \tag{2}$$

$$G \xrightarrow{(\textbf{glucose oxidase})} G + GA + H_2O_2 \tag{3}$$

$$H_2O_2 \xrightarrow{(\textbf{catalase})} H_2O + O_2 + H_2O_2 \tag{4}$$

By assuming that all initial sucrose was hydrolyzed by invertase so the inverted sugar obtained was constituted by equimolecular amount of glucose and fructose, i.e., a mixture containing 50% of each hexose. Then, 1 mol of G present in the inverted sugar would be oxidized by GO generating 1 mol of GA, in the case of complete oxidation of G. Thereby, the final yield (Y), expressed as percentage of glucose oxidized to gluconic acid, was calculated through the equation:

$$Y = 100 - (X - 50) \tag{5}$$

where, X = percent of TRS (fructose plus residual glucose) present in the reaction medium, which is calculated by plotting TRS (%) against time (min), (X−50)=percent of residual glucose.

Multienzymatic Conversion of Sucrose into Fructose and Gluconic Acid (Continuous Process)

The continuous tests were done in CSTR (unimodular-MR, Millipore® ultrafiltration cell) in which a membrane with an MWCO of 100 kDa was placed at the bottom of the reactor vessel. The system presented an air entrance and the reaction volume was set to 300 mL. The internal pressure was kept constant at three bars in all of the experiments by introducing pressurized air along the overall process. The enzymes were added simultaneously and *the exit solution* was collected every hour (100 mL/h) in Erlenmeyer flasks. The amount of invertase, GO, and catalase were varied according to the sucrose concentrations employed (64, 100, or 150 g/L). The temperature was set to 37 °C, the agitation at 300 rpm, pH 5.0 and the residence time at 3 h for all the tests. The amount of residual hydrogen peroxide, glucose, and the total reducing sugars present in each sample of the outlet solution from the membrane reactor was measured. The dissolved oxygen (4 mg/L) in the outlet solution was monitored along the overall process. The inlet solution of sucrose was supplemented with catalase—at an amount of 10% regarding to the initial total activity of the enzyme inside the reactor—at each 3 h. Each catalase addition had duration of 1 h, so that it was carried out in all three supplementations, as the overall duration of the process was 15 h (Fig. 1).

Analytical Determination

Total Reducing Sugar Determination

The TRS was measured by using the Somogyi–Nelson method as described previously [15].

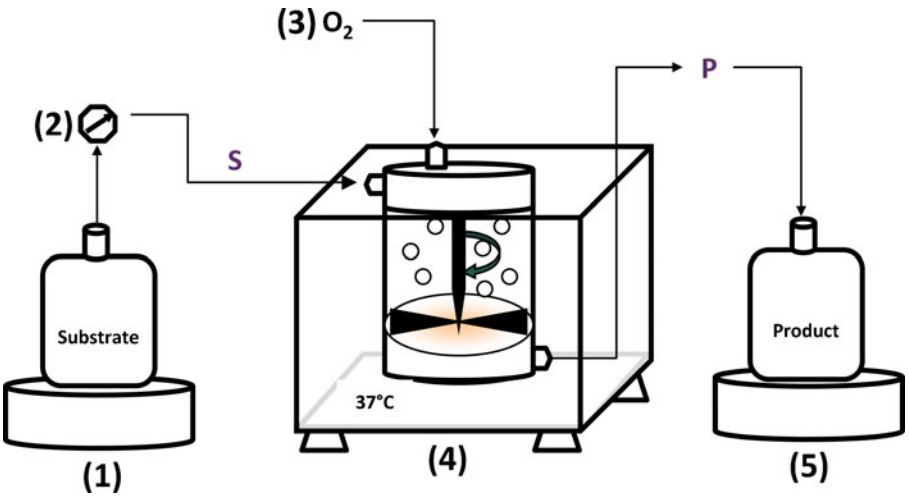

Fig. 1 Scheme of the Continuous process; *1* substrate(sucrose), *2* peristaltic pump, *3* oxygen supply, *4* membrane reactor immersed in a bath, *5* recovered product

Determination of Hydrogen Peroxide

The determination of hydrogen peroxide was made by reading the absorbance at 240 nm. The standard linear equation was $y = 1.6x + 4.90 \times 10^{-2} (r = 0.9994)$, where y and x are the absorbance and the amount of H_2O_2 (mg), respectively. The concentration of the standard solution used to obtain the standard plot was varied between 0.0608 and 0.608 mg of H_2O_2/mL [11].

Determination of Dissolved Oxygen

Dissolved oxygen was measured using a conventional oximeter (DIGIMED, model DM4, São Paulo, Brazil).

Determination of Glucose

The concentration of glucose was measured by using an enzymatic peroxidase/glucose oxidase kit (Laborlab, São Paulo, SP, Brazil).

Results and Discussion

Effect of pH on the Invertase and Glucose Oxidase Activities

Figure 2 shows the variations of invertase and GO activities against pH. The highest activity for invertase and glucose oxidase was observed at pH 4.5 (0.041 mg TRS/min mL) and pH 5.5 (11.5×10^{-4} mg H_2O_2/min mL), respectively. Both pH values are in agreement with those found in the literature [17–20].

As both enzymes are intended to be used simultaneously, in spite of having different pH optima, a compromise for setting the pH of the reaction must be achieved. Comparing the

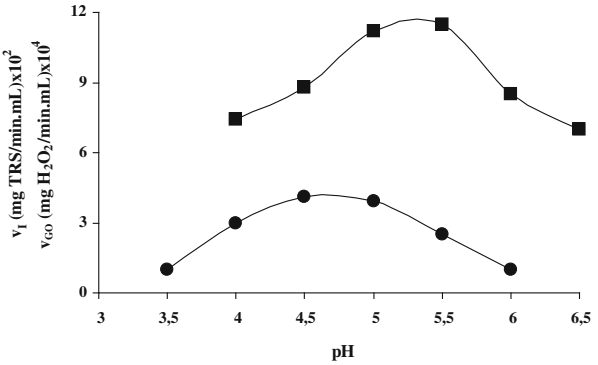

Fig. 2 Variation of the activity of invertase (*filled circle*) and glucose oxidase (*filled square*) against pH

activities of both enzymes at pH 5.0—invertase (0.039 mg TRS/min mL) and GO (11.2×10^{-4} mg H_2O_2/min mL)—resulted that they were about 5% lower than those at 4.5 (invertase) and 5.5 (GO; Fig. 2). Considering 5% variation not significant, so the operational pH was set to 5.0 in all tests performed in batch, fed-batch and continuous-membrane reactors. Besides, the total activity units for both invertase (776.6 U) and GO (7.000 U) effectively employed in the reactor tests have been high enough for compensating any 5% loss.

Invertase, Glucose Oxidase, and Catalase Stability

Table 2 shows the residual activities of invertase, glucose oxidase, and catalase, being the enzymes kept for 30 h at 37 °C and at pH 5.0.

The variation of invertase activity during 30 h was around 2.8% ($\sigma = 0.11\times 10^{-2}$ mg TRS/min mL) and of glucose oxidase was about 14% ($\sigma = 0.17\times 10^{-3}$ mg of H_2O_2/min mL). Considering the time interval between 1 and 30 h, the variation coefficient for glucose oxidase activity was around 8.6% ($\sigma = 0.089\times 10^{-3}$ mg of H_2O_2/min mL). The variation of GO activity observed until t=1 h could be due to a reversible conformational change of its structure (formed by two peptide chains) and/or due to a reversible change of the oxidation-reduction state of its prosthetic groups (Fe^{+2}/Fe^{+3} and $FAD/FADH_2$) [11]. During the same period of time, catalase activity varied around 7% (σ=0.051 mg of H_2O_2/min mL). However, considering the values of the catalase activity at t=0 (0.80 mg H_2O_2/min mL) and at t=30 h (0.67 mg H_2O_2/min mL), an

Table 2 Residual activities regarding to invertase (v_i), catalase (v_{cat}), and glucose oxidase (v_{GO}) maintained for 30 h at 37 °C and at pH 5.0

Time (h)	v_i (TRS mg/min mL)×10^2	v_{GO} (H_2O_2 mg/min mL)×10^3	v_{cat} (H_2O_2 mg/min mL)
0	3.78	1.15	0.80
0.5	4.00	1.41	0.80
1	4.11	1.17	0.77
2	3.96	1.14	0.72
10	3.96	0.96	0.70
20	3.88	0.96	0.68
30	3.83	1.01	0.67

overall diminution of about 16% (Table 2) was observed. A similar behavior for catalase stability against pH was also observed by Wigley [21], who determined that catalase activity at pH 5.0 at 35 °C diminished about 30% after 65 h of incubation.

The study of the kinetics of the catalytic decomposition of hydrogen peroxide is complicated by the fact that while catalase is acting on its substrate, it is being inactivated by this substrate. This also complicates the assay procedures [21]. Thereby, the catalase activity measurement procedure was adapted to the necessities of the present work. As in this work, the catalase is only an auxiliary enzyme, employed in order to maintain the hydrogen peroxide concentration in the reaction medium below 1.3 mM (0.0442 mg/mL), it was enough adapting a kind of exhaustion method. A reaction time of 5 min was set for diminishing the initial hydrogen peroxide concentration up to a value below 1.3 mM or 0, preferably (Fig. 3). The overall reaction time was fixed at 30 min, based on the period of time normally fixed by the cheese-making industry for treating pasteurized milk with catalase in order to eliminate any vestige of peroxide [21].

As observed in Fig. 3, catalase did not convert completely hydrogen peroxide at concentration higher than 0.4 mg/mL under the reaction conditions used in this work. Such result would be due to the enzyme inactivation by the substrate, as already commented. Moreover, the slopes of the curves between 0 and 5 min can be taken as the catalase activity against the correspondent initial hydrogen peroxide concentration, whose values are shown in Table 3. By applying the conventional Lineweaver–Burk plot to the data presented in Table 3, the kinetic constants for catalase, under the conditions employed here, could be estimated. Thereby, from Fig. 4 Km and V_{max} were estimated as 4.03 mg/mL and 0.86 mg H_2O_2 consumed/min mL, respectively. A comparison of these values with those found in the literature is quite difficult because of the large variety of procedures employed for measuring the catalase activity. Just for comparison, the Km and V_{max} calculated by Wigley [21] to bovine catalase were equal to 2.4 mg/mL and 10.6 mg H_2O_2 consumed/min mL, respectively.

Here, the data presented above suggest the need to circumvent the substrate inactivation of catalase, mainly when it is intended to use a multienzyme system, as in the present work, constituted by invertase, GO, and catalase, for converting sucrose solution at concentration

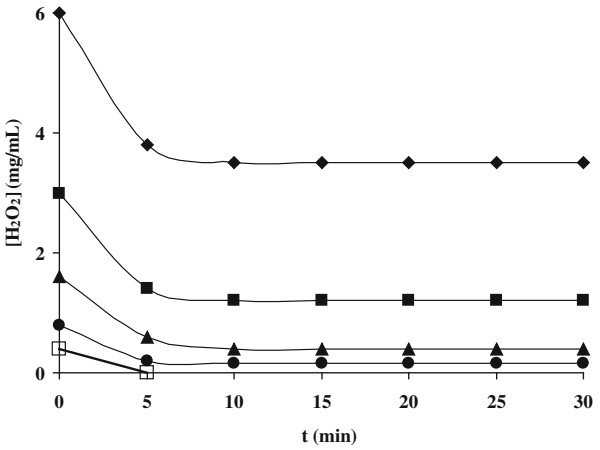

Fig. 3 Consumption of hydrogen peroxide along the reaction catalyzed by catalase. The initial concentration of substrate was: 6.0 mg/mL (*filled diamond*), 3.0 mg/mL (*filled square*), 1.6 mg/mL (*filled triangle*), 0.8 mg/mL (*filled circle*), and 0.4 mg/mL (*empty square*)

Table 3 Catalase activity (v_{cat}) against initial hydrogen peroxide concentration for applying the Lineweaver-Burk plot in order to estimate the kinetic constants of the enzyme

$[H_2O_2]$ (mg/mL)	$1/[H_2O_2]$ (mL/mg)	[a] v_{cat} (mg H_2O_2/mL min)	$1/v_{cat}$ (mL min/mg H_2O_2)
0.2	5.0	0.04	25.0
0.4	2.5	0.08	12.5
0.8	1.25	0.14	7.14
1.6	0.625	0.24	4.17
3.0	0.333	0.36	2.78
6.0	0.167	0.48	2.08

[a] Calculated according to Eq. 1

up to 150 g/L. In such case, it would be expected near 75 g/L of overall hydrogen peroxide to be decomposed by catalase. Of course, such a high concentration of hydrogen peroxide could completely be adverse to the catalase catalysis.

However, if it is considered that H_2O_2 would appear in the medium stepwise instead of at full amount, so catalase could be added to the reaction medium at fed-batch mode. Such an approach was applied, being the data of one assay plotted in Fig. 5. As shown, after 20 min of reaction, the amount of H_2O_2 present into the medium was practically zeroed. Thereby, this approach was set as pattern for carrying out any fed-batch or continuous process in which the conversion of sucrose to fructose and gluconic acid or glucose to gluconic acid is the main goal. The partitioned addition of catalase could lead to the diminution of the overall bubbling inside the reactor, which, in turn, would avoid the splashing of the reaction medium from the vessel as well as the obstruction of the connecting tubes of the reactor.

Sucrose Hydrolysis by Invertase (Batch Process)

Sucrose hydrolysis, which is about 30 times faster than the glucose oxidation by GO (Table 2), will not be the limiting step, when both enzymes would act simultaneously on sucrose for attaining the fructose and gluconic acid. Thereby, the overall duration of sucrose hydrolysis might be as low as possible, in order to leave the overall conversion to be governed by the glucose oxidation step. As it is shown in Fig. 6, the complete sucrose (64 g/L) hydrolysis by invertase (776.6 U) occurred after 50 min reaction.

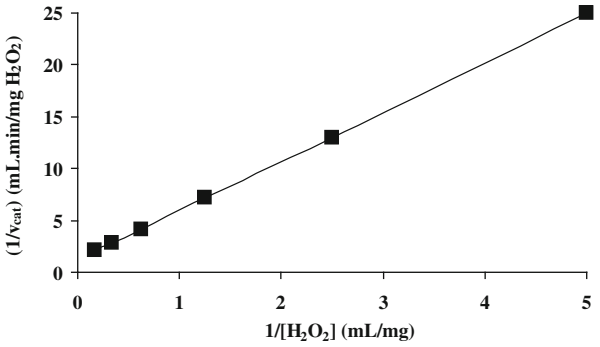

Fig. 4 Lineweaver–Burk plot for estimating the kinetic constants of catalase

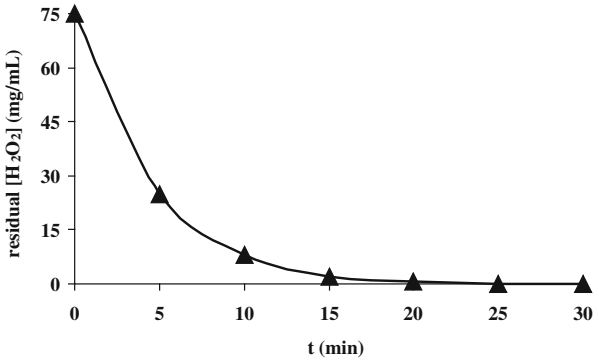

Fig. 5 Consumption of hydrogen peroxide by catalase, in which both enzyme (2,500 U/mL) and substrate (75 mg/mL) solutions have been added into the reaction medium (at intervals of 5 min) as aliquots of 4 and 6 mL, respectively. The whole volumes of catalase and hydrogen peroxide solutions added to the medium were 24 and 30 mL, respectively

Sucrose Hydrolysis and Glucose Oxidation (Fed-Batch Process)

Table 4 presents the percentages of sucrose conversion by invertase in a fed-batch reactor against different substrate solution addition laws into the reactor. It is observed that sucrose hydrolysis was complete when the substrate was added according to the linear and exponential decreasing laws. However, the average of hydrolytic activity for the linear decreasing addition was 12% higher than the one obtained with the exponential decreasing addition. Probably, in the decreasing linear addition condition, a larger amount of substrate was available to invertase during the reaction than the one of exponential decreasing addition. Furthermore, the total substrate conversion took place at t=55 min (Fig. 7), so quite similar to the one observed in the discontinuous process.

It can be seen from Fig. 8 that the oxidation of glucose was not complete in spite of a sharp decreasing of glucose clearly observed up to 25 min of reaction. However, the highest conversion yield (72%) occurred when the glucose solution (32 g/L) has been added according to the linear decreasing law (Table 5). This result could be explained by the presence of hydrogen peroxide into the reaction medium, which over 1.3 mM can inhibit

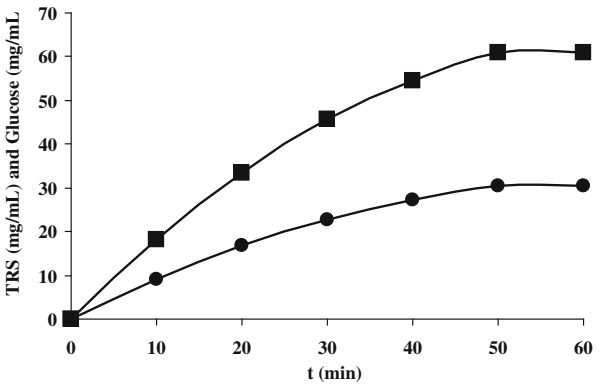

Fig. 6 Total reducing sugars formation (*filled square*) against time for the hydrolysis of 64 g/L sucrose solution. The accumulation of glucose (*filled circle*) into the reaction medium was also followed

Table 4 Average hydrolysis activity of invertase on 64 g/L of sucrose solution added to the reactor according to different addition laws

The conversion yield of the fed-batch tests regarding to each type of addition were also presented

Feeding type	*v* (TRS g/min)	Yield (%)
Constant	0.29	92.5
Linear decreasing	0.40	100.0
Linear increasing	0.33	79.6
Exponential decreasing	0.35	100.0
Exponential increasing	0.30	91.9

not competitively the glucose oxidase [11]. Besides, the average rate of glucose oxidation, when the substrate was added according to the linear decreasing law, was about 13% higher than that attained when the addition law followed the exponential decreasing pattern (Table 5). This result is similar to that found for sucrose hydrolysis by invertase (Table 4). Therefore, the linear decreasing addition mode has been set to carry out the fed-batch test, in which the conversion of sucrose to fructose and gluconic acid has been conducted by employing invertase and GO simultaneously.

Multienzyme Conversion of Sucrose into Fructose and Gluconic Acid (Fed-Batch Process)

As discussed previously, the fed-batch process was done by adding the sucrose solution according to the linear decreasing law. The reactor was filled from 100 up to 400 mL within 4 h. In Fig. 8, the profile of sucrose conversion into fructose and gluconic acid in terms of TRS accumulation in the reaction medium is shown. The residual glucose present in the medium was also followed along the assay (Fig. 8).

Figure 9 shows that 64% of TRS remained into the medium at the end of the reaction (X). By introducing X=64% in Eq. 5, results Y=86%, which corresponded to the yield of G/GA conversion by GO. The incomplete glucose conversion (X>50%) would have resulted from the unbalancing between hydrolytic and oxidative reactions. As mentioned beforehand, the hydrolysis was about 30-fold faster than oxidation resulting in the filling

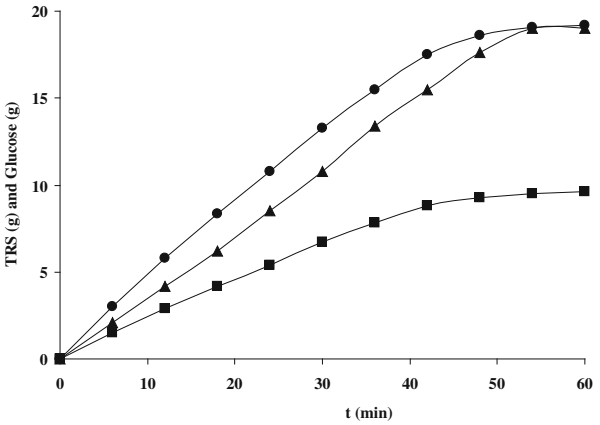

Fig. 7 TRS formation during fed-batch processes where a sucrose solution (64 g/L) was added according to the following laws: linear decreasing (*filled circle*) and exponential decreasing (*filled triangle*). The formation of glucose along the reaction was also determined as sucrose was added according to linear decreasing law (*filled square*)

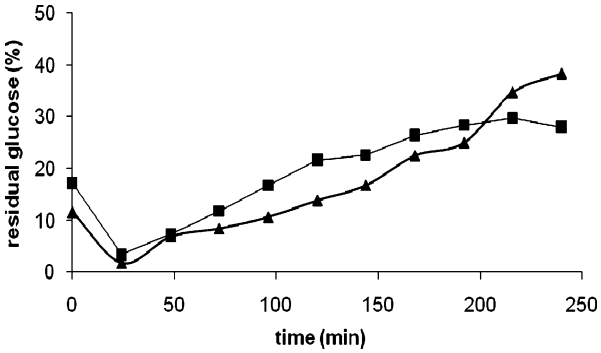

Fig. 8 Profiles of glucose conversion during fed-batch processes where a glucose solution (32 g/L) was added according to the following laws: linear decreasing (*filled square*) and exponential decreasing (*filled triangle*)

time of 4 h which was not enough for GO to convert all the glucose present into the reaction medium. Moreover, the incomplete oxidation could also arise from the fact that the catalase activity into the medium was not enough to decompose the hydrogen peroxide formed, which, in turn, accumulated into the medium surpassing the inhibitory concentration (1.3 mM) on GO activity (Table 6).

Multienzyme Sucrose Conversion in Fructose and Gluconic Acid (Continuous Process)

The multienzyme continuous sucrose conversion percentages are shown in Table 7. The initial sucrose concentration was 64, 100, or 150 g/L and different activities of enzymes (invertase, GO, and catalase) were used. All the tests were carried out for 15 h and their dilution rate was kept constant (0.33 h^{-1}).

From Table 7, it may be noticed that the amount of the enzymes should be adjusted in function of the initial concentration of sucrose to be introduced into the reactor. By comparing tests 1 and 2, a reduction of about ten times in the total invertase activity resulted in a twofold increase in glucose conversion yield. In test 1, whose invertase activity was high, the overall dynamic process might have been affected, once GO oxidative activity did not follow the glucose arising from sucrose hydrolysis. The longer stationary state, however, was observed in test 2, which was reached 6 h after starting the continuous substrate feeding into the reactor (Fig. 10).

When the concentration of the sucrose solution was varied from 64 to 100 g/L (test 3), the transient regime inside the membrane reactor lasted for 15 h. As consequence, the conversion yield could not be calculated (Table 7). Certainly, the amount of invertase inside

Table 5 Average oxidative activity of glucose oxidase on 32 g/L of glucose solution added to the reactor according to different addition laws

Conversion yields of the fed-batch tests regarding to each type of addition were also presented

Feeding type	*v* (glucose conv/min)	Yield (%)
Constant	0.023	57.22
Linear decreasing	0.029	72.00
Linear increasing	0.017	42.82
Exponential decreasing	0.025	61.70
Exponential increasing	0.024	60.14

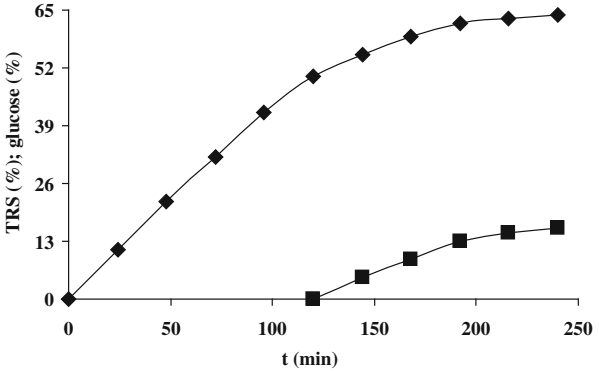

Fig. 9 Percent of TRS accumulated against time for the [sucrose/(fructose + gluconic acid)] conversion by a fed-batch process, in which invertase, GO, and catalase acted simultaneously (*filled diamond*). Residual glucose present into the medium along the reaction was also determined (*filled square*). The sucrose solution was added according to the linear decreasing law (Table 2)

the reactor was not enough to convert all the sucrose introduced. In test 4, the invertase activity was increased to 176.5 U, whereas the catalase activity was augmented to 295,000 U, in order to match the hydrogen peroxide increasing due to the use of more concentrated sucrose solution (100 g/L). At the end, a yield of 67.4% in test 4 was reached (Table 7).

Aiming the improvement of the sucrose conversion at an initial concentration of 100 g/L, the test 5 was designed by maintaining the invertase activity at 176.5 U and increasing the GO and catalase activities to 9,000 and 354,000 U, respectively. A yield of about 83% was achieved (Table 7) and the steady-state was reached after 8 h of continuous feeding (Fig. 10).

As the previous result indicated that a probable increase on the GO and catalase activities should increase the yield of sucrose conversion at the concentration of 100 g/L, a more concentrated sucrose solution, i.e., 150 g/L, was tried. Therefore, test 6 was carried out at initial sucrose concentration of 150 g/L; invertase, GO, and catalase activities equal to 264.75, 13,000, and 531,000 U, respectively. As shown in

Table 6 Residual hydrogen peroxide present in the reaction medium during the multienzyme conversion of sucrose through fed-batch process

Time (min)	H_2O_2 (mM)
0	0
24	0
48	0
72	0
96	0.5
120	1.1
144	2.0
168	2.5
192	3.0
216	3.2
240	3.0

The substrate solution was added into the reactor according to the linear decreasing law

Table 7 The yield of glucose oxidation to gluconic acid reached through continuous sucrose conversion conducted in a membrane reactor by using different activities of invertase, GO, and catalase

Test *n*°	D (h^{-1})	sucrose (g/L)	Enzymatic activity (U) Invertase	GO	Catalase	Glucose oxidation yield (%)
1	0.33	64	776.6	7,000	236,000	43.9
2	0.33	64	70.6	7,000	236,000	92.3
3	0.33	100	70.6	7,000	236,000	–
4	0.33	100	176.5	7,000	295,000	67.4
5	0.33	100	176.5	9,000	354,000	83.0
6	0.33	150	264.75	13,000	531,000	76.0

Fig. 10, the steady-state was reached after 10 h of continuous feeding, the conversion yield being about 76% (Table 7).

The importance of working with concentrated sucrose solutions, above 150 g/L, is due to the fact that the sugarcane molasses, a residue of sugar production, possesses average sucrose contents of about 300 g/L [22]. In order to take advantage of the sugarcane molasses, as a source of sucrose, it should be diluted in 50% at the most to avoid the manipulation of a great volume of the substrate.

Following the exposed consideration above, an optimization of the conversion achieved in test 6 was attempted by increasing the catalase activity (649,000 U; data not shown). Nevertheless, the process was unflattering due to an intense foaming, which resulted from the decomposition of hydrogen peroxide that hindered the maintenance of its entrance and exit flows constant.

Figure 11 displays the formation of H_2O_2 during the continuous feeding of the membrane reactor (tests 2, 5, and 6), in which highest yields have been attained (Table 7).

By comparing the highest amount of H_2O_2 formed in the multienzymatic fed-batch test (linear decreasing flow; Table 6), approximately 3.0 mM after 4 h, with the one formed in the continuous test (test 2), about 1.5 mM, the concentration of H_2O_2 was the probable reason for the smallest yield obtained in the fed-batch process, inasmuch in both cases the sucrose solution used was of 64 g/L.

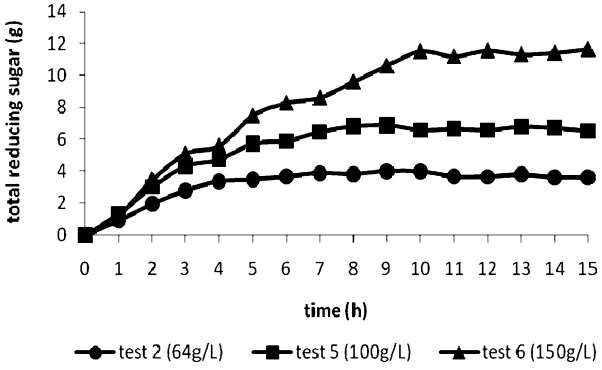

Fig. 10 TRS measured during continuous membrane reactor fed with sucrose at concentrations of 64 g/L (*filled circle*), 100 g/L (*filled square*), and 150 g/L (*filled triangle*), which corresponded to test 2, 5, and 6, respectively

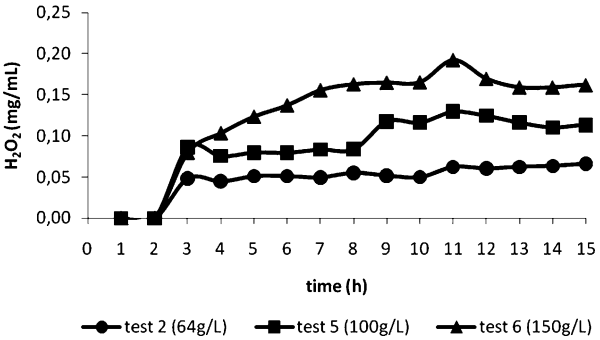

Fig. 11 Formation of H_2O_2 during the tests performed in the continuous membrane reactor, which presented highest yields. Test 2 (*filled circle*), test 5 (*filled square*), and test 6 (*filled triangle*)

Conclusions

The obtained results allowed the conclusion that the invertase and GO had good activity at pH 5.0, although their optimum pH are 4.5 and 5.5, respectively. Besides, they presented good stability, for at least 30 h at pH 5.0 and at 37 °C. The same occurred to catalase. The addition of the substrate solutions (sucrose and glucose) according to a linear decreasing law, allowed obtaining the highest conversion: 100% in sucrose hydrolysis by invertase and 72% in glucose oxidation by GO and catalase. In multienzymatic conversion of sucrose (64 g/L) by using the fed-batch process and the continuous process (membrane reactor), the obtained yields were around 84% and 92%, respectively. In addition, it yields around 83% and 76% were attained when the membrane reactor has been fed with sucrose solution of 100 and 150 g/L, respectively. It must be borne out that the catalase had to be added in portions when the reaction was conducted either as fed-batch or continuous mode.

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