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ENTRAPMENT OF INVERTASE AND GLUCOSE OXIDASE IN ACRYLAMIDE MATRIX

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ABSTRACT

Invertase and glucose oxidase were immobilized by entrapment in polyacrylamide hydrogel. The support was electrically neutral as $pH_{optimum}$ shifting was not observed for both enzymes. The concentration of the cross-linking agent (EDMA) played a crucial role in the immobilization coefficient (IC), which was over 30% for both enzymes. The concentrations of IC and EDMA were linearly correlated. Invertase and glucose oxidase (GO), either soluble or immobilized, were highly stable when used in a membrane bioreactor operated continuously for at least 30 h. All enzymes forms were thermodynamically characterized and the following kinetic constants were calculated: **a) Invertase: a.1) soluble form:** V_{max} = 84 g RS/min. g protein; K_M = 22.6 mM; E_a = 37.0 kJ/mol; ΔG = -10.5 kJ/mol; ΔH = 34.4 kJ/mol; ΔS = 0.141 kJ/mol.K; **a.2) immobilized form:** V_{max} = 65 g RS/min. g protein; K_M = 17.0 mM; E_a = 24.3 kJ/mol; ΔG = - 10.6 kJ/mol; ΔH = 21.7 kJ/mol; ΔS = 0.102 kJ/mol.K; **b) Glucose oxidase: b.1) soluble form:** V_{max} = 0.218 ΔA/min; K_M = 7.89 mM; E_a = 28.0 kJ/mol; ΔG = - 11.4 kJ/mol; ΔH = 25.4 kJ/mol; ΔS = 0.180 kJ/mol.K; **b.2) immobilized form:** V_{max} = 0.144 ΔA/min; K_M = 7.69 mM; E_a = 53.5 kJ/mol; ΔG = - 11.0 kJ/mol; ΔH = 50.9 kJ/mol; ΔS = 0.20 kJ/mol.K.

KEYWORDS: Entrapment, acrylamide, glucose oxidase, invertase.

INTRODUCTION

Invertase (E.C.3.2.1.26) and glucose oxidase (E.C.1.1.3.4) are enzymes intensely studied and used in the industry and in analytical procedures along the last sixty years. Invertase and

glucose oxidase were described in 1860 by Berthelot and in1904 by Maximow, respectively.[1,2]

Invertase catalyzes sucrose hydrolysis, resulting in glucose and fructose. This mixture is either marketed as inverted syrup (used as sweetener in the food industry) or as a source for obtaining pure glucose and fructose, marketed as analytical-grade reagents. This enzyme has also been used as medicine (as antimicrobial and antioxidant agent), in probes for analytical measurement of sucrose, and in ELISA kits to detect yeasts of the genus *Candida*. [2] Invertase has been extracted and purified (purification factor of approximately 7) mainly from cells of Saccharomyces cerevisiae (residue of the ethanol industry) and *Saccharomyces carlsbergensis* (residue of the brewing industry). [3][4][5] Moreover, sucrose hydrolysis has also been explored by using intact yeast cells due to the presence of invertase in the cell wall. This can be considered as an example of "natural immobilization".[6]

Glucose oxidase (GO) – obtained from strains of *Aspergillus niger* and *Penicillium amagasakiense* – catalyzes D-(+)-glucose oxidation in gluconic acid and hydrogen peroxide. However, there are two isomeric forms of D-(+)-glucose in aqueous solution – α and β forms, also called anomers; the phenomenon is called mutarotation–, with GO being specific for the β form. This practically does not constitute a problem for complete glucose oxidation by GO, as α and β forms are in equilibrium when D-(+)-glucose is dissolved in water.[7] GO is used in biosensors (enzyme electrodes – amperometric and potentiometric types – for glucose measurement), controlling devices (paper strips for glucose detection in the urine of diabetic patients, for example), clinical diagnosis (in auto-analysers and immunoassays), basic research (studies on the constitution and catalytic mechanism of flavoprotein dehydrogenases, for instance), chemical industry (synthesis of gluconic acid and fructose separation from inverted syrup), and in the food industry (to desugaring eggs – egg white, yolk, or whole egg – to avoid the Maillard reaction; and as antioxidant in juices, beer, and wine by reducing the amount of oxygen dissolved in these products).

Recently, combined use of invertase and glucose oxidase has been proposed for directly converting sucrose into gluconic acid and fructose by using a membrane bioreactor operated in continuous mode.[8]

The immobilization technique resulted from the empirical observation of how thousands of enzymes are wrapped inside small dimension cells. Moreover, enzymes inside the cell have high catalytic performances. This occurs as enzymes are interacting with membranes (belonging to organelles such as the endoplasmic reticulum, Golgi complex, and cytoskeleton, etc.), i.e., being naturally immobilized. Thereby, researchers had the idea to bind enzymes in inert supports, resulting in the following immobilization techniques: BINDING IMMOBILIZATION **[binding to supports** (ionic, adsorptive, and covalent binding); and **cross-linking** (cross-linking and co-cross-linking)**];** and **PHYSICAL IMMOBILIZATION [matrix entrapment (beads and fibers); and membrane enclosure** (encapsulation and membrane reactors)**].**[9]

This study evaluates the parameters of fiber-type matrix entrapment (catalyst amount, temperature, pH, concentration of cross-linking agent, and polymerization starters, etc.) for both enzymes and the operational stability of soluble and immobilized enzymes in a membrane bioreactor operated in continuous mode.

MATERIAL AND METHODS

Material

Invertase and glucose oxidase were purchased from MERCK and SIGMA, respectively. The 20-kDa UF membrane was purchased from MILLIPORE and all other reagents were of P.A. grade. A 500-mL pressure-resistant-glass membrane bioreactor, purchased from Millipore®, containing a 20-kDa UF membrane at the bottom (cellulose acetate membrane DDS 600) was used.

Methods

Analytical Techniques

Measurement of soluble protein

Soluble protein concentration was determined by using the Lowry method.[10]

A bovine albumin solution (0.83 mg/mL) was used to determine the standard minimum square linear regression equation:

$Y_p = 0.002175.X_p - 0.002$ $(r = 0.995)$ (Eq. 1)

Where Y_p = absorbance read at $\lambda = 660$ nm; X_p = protein amount (μg).

Measurement of invertase activity

In a test tube, 3.2 mL of acetate buffer (50 mM, pH 4.6) and 0.8 mL of sucrose solution (0.3 M) were added. The tube was immersed for 5 min in a water-bath at 37 ^{o}C. Subsequently, a volume (5 – 60 μL) of invertase solution (2 μg/mL) was added and the reaction was left for 15 min. Afterwards, a 1-mL aliquot was collected and mixed with 1 mL of the alkaline cupric reagent of Somogyi, with the tube being immersed in boiling water for 20 min. After cooling, 1 mL of arsenic-molybdenum reagent and 7 mL of distilled water were added. The color developed was read at λ = 520 nm in a spectrophotometer.[2]

For determining immobilized invertase activity, acetate buffer (3.2 mL) and invertase powder (5.0 mg) were mixed in a test tube, followed by immersion in a water-bath at 37 ^{o}C for 30 min under agitation (50 rpm). Subsequently, 0.8 mL of sucrose solution (0.3 M) was added and the reaction was left for 15 min. After that, the procedure was the same aforementioned, except that the colored suspension was centrifuged (3,000*g*/5 min) before being read in the spectrophotometer.

Invertase activity was expressed as absorbance/min.μg of enzyme. Correlation between absorbance and invertase amount (μg) was established by conducting the reaction and changing invertase amounts in the interval of 0.01-0.12 μg. The standard minimum square linear regression equation was:

$Y_i = 5.74.X_i + 0.002$ (r = 0.9994) (Eq. 2)

Where Y_i = absorbance read at λ = 520 nm; X_i = invertase amount (μg).

Measurement of glucose oxidase (GO)

In a test tube, 2.775 mL of 50 mM acetate buffer (pH = 5.1) and 0.125 mL of 56 mM glucose were added and maintained under agitation of 50 rpm for 5 min at 37 ^{o}C. Then, 0.5 mL of glucose oxidase solution (1 mg/mL) was added, followed by (after 5 min of reaction) immersion of the tube in boiling water for 2 min. Then, a 0.1-0.5-mL aliquot was transferred to a 3 mL-cuvette containing 2.4 mL of o-dianisidine (5 mM) and 0.05 mL of peroxidase (0.5 μg/mL). Color development was followed in a spectrophotometer (λ = 436 nm) at 37 ^{o}C with registration speed equal to 30 mm/min and maximum absorbance set at 0.500. For the immobilized GO, the content of the test tube was centrifuged (5,000*g*/5 min) after cooling and before photometric measurement.

Soluble GO activity was calculated by equation:

$C = (1.36.a) \div m$ (Eq. 3)

Where: C = activity [(ΔA/min)/min. mg GO]; m = GO amount (mg); a = absorbance variation during 1 min of reaction (ΔA/min).

Immobilized GO activity was calculated by equation:

$C_1 = (0.113.a_1) \div m_p$ (Eq. 4)

Where: C_1 = activity expressed as [(ΔA/min)/min.mg of polymer]; a_1 = absorbance variation during 1 min of reaction (ΔA/min); m_p = polymer amount (mg).

Enzyme Immobilization

In a 50 mL-beaker, 6 mL of 2-hydroxymetacrylate (HEMA), ethylene-dimetacrylate (EDMA), and 6 mL of distilled water were poured. The beaker was bubbled with nitrogen for 15 min and the mixture was left at 4 ^{o}C. Subsequently, the enzyme and the starter reagent were added and, after mixing, the solution was poured into a Petri dish (diameter = 20 cm), followed by water bath at 4 ^{o}C and exposure to UV light (365 nm) for 1 h. The polymer formed was rinsed with distilled water and left in a desiccator containing silica gel as desiccating agent until complete drying of the polymer. Afterwards, the polymer was crushed and sieved. Particles between 65 and 115 mesh were separated. Polymerization parameters changed for each enzyme are shown in Table 1.

Table 1: Parameters changed for invertase and glucose oxidase immobilized in acrylamide matrix.

Parameter	Invertase	Glucose oxidase
Enzyme amount (μg)	4, 15, 30, 45, 60, and 90	4, 8, 16, and 32
Polymerization temperature (^{o}C)	4 and 20	4 and 10
EDMA concentration (% v/v)*	0.5, 1, 2, 4, and 5	0.5, 1. 2, and 4
Starter type	($FeSO_4$ + H_2O_2)	Benzoin methyl ether (BME)
Starter concentration (M)	($FeSO_4$): between $0.5x10^{-3}$ and $6.25x10^{-3}$ (H_2O_2): between $2x10^{-3}$ and $5x10^{-3}$	Between $1x10^{-3}$ and $1x10^{-4}$
Type of polymerization adjuvant	PEG 1500 and PEG 6000	PEG 1500 and PEG 6000
Adjuvant concentration (% p/v)	1 and 2	1 and 2

*The percentage is calculated regarding the amount of HEMA used.

The immobilization efficiency in acrylamide matrix for invertase (R_I) was calculated by equation:

$R_I = 100. (m_1/m.m_2).P. (\Delta A_a/\Delta A_p)$ (Eq. 5)

Where: ΔA_a = change in absorbance for immobilized invertase; ΔA_p = change in absorbance for soluble invertase; P = polymer dry weight (μg); m = initial amount of invertase (μg); m_1 = invertase amount related to ΔA_p (μg); m_2 = amount of immobilized invertase related to ΔA_a (μg).

The immobilization efficiency in acrylamide matrix for glucose oxidase (R_{GO}) was calculated by equation:

$R_{GO} = 100. (P.C_1) \div (C.m_1)$ (Eq. 6)

Where: C_1 = immobilized GO activity [(ΔA/min)/min.mg of polymer]; C = soluble GO activity [(ΔA/min)/min. mg GO]; P = dry matter of the complex of GO and acrylamide (mg); m_1 = GO amount (mg).

Enzyme Characterization

Determination of kinetic parameters

Kinetic parameters (K_M and V_{max}) were calculated by Hanes-Woolf method.

Invertase activity was measured at 37 ^oC and pH = 5.0 for 15 min against changing in sucrose concentration from 2.5 to 200 mM. The procedure followed that described above.

Glucose oxidase activity was measured at 35 ^oC and pH = 5.1 for 3 min against changing in β-D-glucose concentration from 0.30 to 1.6 mM. The procedure followed that described above.

Activity and stability against temperature

Activity was measured by changing the temperature between 30 ^oC and 55 ^oC. Stability was measured by leaving the enzyme solution at a desired temperature (30 – 60 ^oC) for 30 min. Then, the tube was immersed in water bath at 37 ^oC and 35 ^oC for invertase and GO, respectively, and residual activity was measured as described above.

Effect of pH on enzyme activity

The effect of pH on invertase and GO activity was evaluated against changing in pH from 4.0 to 6.0. The procedure followed that described above.

Continuous Process

Sucrose hydrolysis by invertase and glucose oxidation by GO were performed in a 500-mL pressure-resistant-glass membrane reactor containing a 20-kDa UF membrane at the bottom.[11] Sucrose and glucose were added continuously to the reactor, with the solution being saturated with nitrogen (sucrose) and oxygen (glucose). Gas saturation was required as O_2 is a substrate for GO and nitrogen is an inert gas that avoids glucose oxidation after sucrose hydrolysis. Samples were collected every hour, in which reducing sugars and hydrogen peroxide content were measured.[11]

RESULTS AND DISCUSSION

Invertase

The kinetic constants for soluble and immobilized invertase were calculated by Hanes-Woolf plot (Figure 1).

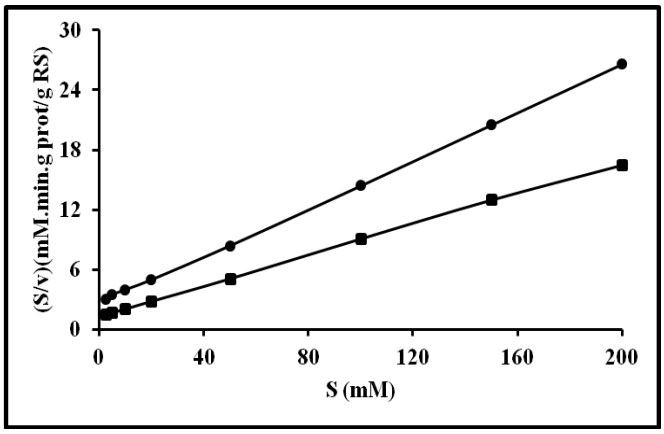

Figure 1: Hanes-Woolf plot for calculating the kinetic constants of soluble (●) and immobilized (■) invertase. Ordinate values must be divided by 10 and 5 for soluble and immobilized invertase, respectively.

Standard minimum square linear regression equations are:

$(S/v)_s = 0.269 + 0.0119.(S)$ (r = 0.9998) (Eq. 7)

$(S/v)_i = 0.262 + 0.0154.(S)$ (r = 0.9995) (Eq. 8)

Where: (S) = substrate concentration (mM); $(S/v)_s$ and $(S/v)_i$ refer to soluble and immobilized invertase, respectively.

Thereby, kinetic constant values were: $(V_{max})_s$ = 84 g RS/min. g protein; K_{Ms} = 22.6 mM; $(V_{max})_i$ = 65 g RS/min. g protein; and K_{Mi} = 17.0 mM.

As observed, K_{Mi} was approximately 25% lower than K_{Ms}, while $(V_{max})_i$ was approximately 23% lower than $(V_{max})_s$. Difference among kinetic constants between soluble and immobilized catalysts is a general rule in this field. However, $K_{Mi} < K_{Ms}$ has been observed for immobilization by entrapment, especially for hydrogel encapsulation, in which the enzyme-substrate interaction is not intensely affected by diffusion of low molecular mass (MW) molecules through the gel membrane. [12][13] Unlike K_M, $(V_{max})_i < (V_{max})_s$ is a common result because – as the kinetics of enzyme catalysis states that $V_{max} = k.(E)$, i.e., the maximum activity is proportional to the enzyme concentration (E) [14] – not all enzyme molecules entrapped are available to the substrate (steric hindrance, for example) and/or have the adequate conformation for catalysis. The latter, for instance, is verified to invertase, that requires an aggregation of four molecules to reach the highest activity. [15]

Effect of pH on both invertase forms is shown in Figure 2.

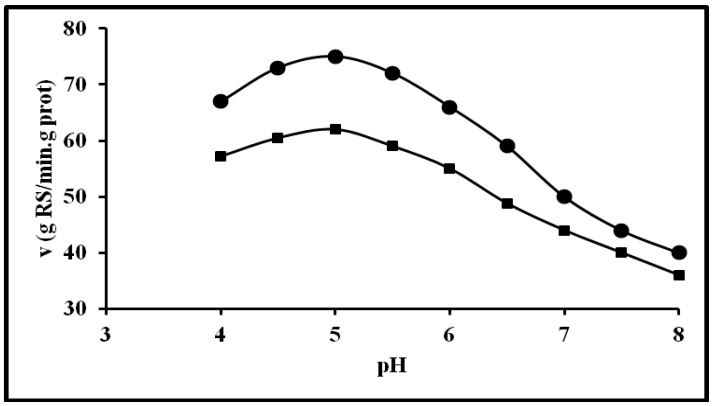

Figure 2: Effect of pH on the activity of soluble (●) and immobilized (■) invertase.

Both invertase forms had $pH_{optimum}$ at 5.0. This result indicates that the acrylamide gel is electrically neutral, which implies that the lattice structure of the matrix – the microenvironment surrounding invertase molecules – do not partitions ionized molecules between the microenvironment and the bulk solution, a phenomenon that could decrease enzyme activity. Moreover, sucrose, the preferential invertase substrate, is not a charged molecule, as well as the products (glucose and fructose), so their movement through the gel is

only ruled by diffusion, which as aforementioned, has decreased interference on hydrolysis rate.

The effect of temperature on the activity of both invertase forms is presented in Figure 3. Considering the temperature interval from 318K to 333K, the activation energy (E_a) for soluble and immobilized invertase was calculated by Arrhenius method with the standard minimum square linear regression equations:

$\text{Ln } v_s = -9.651 + 4446.(1/T)$ $(r = 0.9991)$ (Eq. 9)

$\text{Ln } v_i = -4.984 + 2920.(1/T)$ $(r = 0.996)$ (Eq. 10)

Where: T = absolute temperature (K); Ln v_s and Ln v_i being the natural logarithms of the activities of soluble and immobilized invertase, respectively.

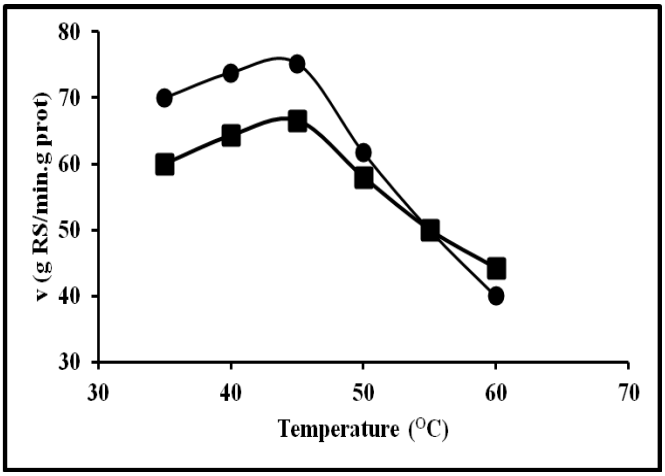

Figure 3: Effect of temperature on the activity of soluble (●) and immobilized (■) invertase.

Table 2 shows the values of thermodynamic parameters (ΔG, ΔH, ΔS) regarding both invertase forms, which were calculated by equations:

$\Delta G = (R.T/2.303).\text{Log } (v.h/k.T)$ (Eq. 11)

$\Delta H = E_a - R.T$ (Eq. 12)

$\Delta S = (\Delta H - \Delta G)/T$ (Eq. 13)

Where: ΔG = Gibbs free energy (kJ/mol); ΔH = enthalpy (kJ/mol); ΔS = entropy (kJ/mol.K); R = 8.3144 (J/K.mol); T = absolute temperature (K); v = enzyme activity; k = 1.38×10^{-23} (J/K); h = 3.978×10^{-32} (J.min).

Table 2: Thermodynamic parameters related to soluble and immobilized invertase. The temperature was set at 318K.

Enzyme	E_a (kJ/mol)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/mol.K)
Soluble	37.0	- 10.5	34.4	0.141
Immobilized	24.3	- 10.6	21.7	0.102

Data in Table 2 showed that the spontaneity of the process, represented by ΔG, has not been changed by immobilization, while ΔH and E_a decreased for immobilized invertase, indicating that hydrolysis requires less energy than with the use of soluble invertase. Entropy decreased approximately 28% for immobilized invertase, indicating that immobilization led to a system structurally more organized.

The stability of immobilized invertase against temperature was higher than the stability of soluble invertase (Figure 4). This result corroborates the rules of immobilization, which state that the procedure commonly leads to enzyme stabilization.

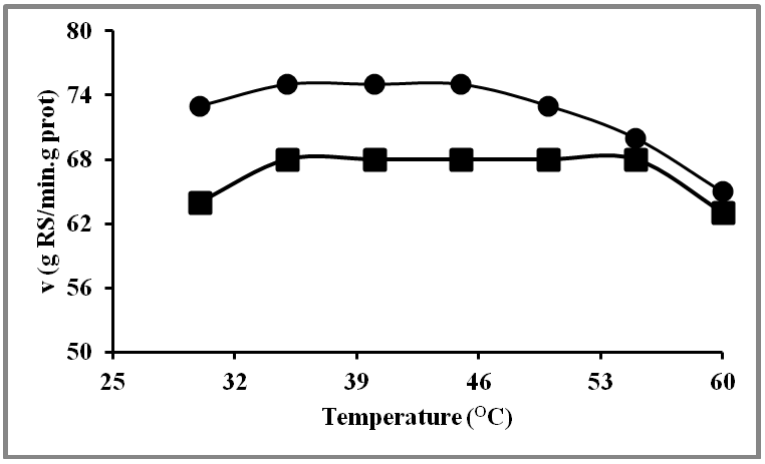

Figure 4: Effect of temperature on the stability of soluble (●) and immobilized (■) invertase.

The best immobilization conditions in acrylamide hydrogel were observed for changes in the amount of cross-linking agent (EDMA: 0.5%, 1%, 2%, and 4%), the enzyme amount (5.0, 15, 45, 60, and 90 mg), and for the type (PEG 1500 and PEG 6000) and amount of adjuvants (1% and 2%). Concentrations of $FeSO_4$ ($1.25x10^{-3}$ M) and H_2O_2 ($5.0x10^{-3}$M), as well as temperature (set at 4 ^oC) were maintained unchanged, except for test 6, performed at 20 ^oC. Data is shown in Table 3.

Table 3: Effect of procedure conditions on the immobilization coefficient (IC) of invertase in polyacrylamide hydrogel.

Test	Variation of parameter	Polymerization time (min)	IC (%)
1	EDMA: 2%	90	23
2	EDMA: 4%	90	7.0
3	EDMA: 1%	80	34.5
4	EDMA: 0.5%	105	36.1
5*	Invertase: 5 mg	150	6.7
6***	Temperature: 20 ^{o}C	48	22.5
7*	Invertase: 15 mg	180	22.7
8*	Invertase: 60 mg	110	25.8
9*	Invertase: 45 mg	120	27.5
10*	Invertase: 90 mg	75	11.3
11**	PEG 6000: 1%	80	26.7
12**	PEG 6000: 2%	53	22.2
13**	PEG 1500: 1%	70	26.1
14**	PEG 1500: 2%	90	21.5

*Tests performed without adjuvant and at EDMA = 1%; **Tests performed at invertase = 30 mg and EDMA = 1%; ***Test performed at EDMA = 1%, invertase = 30 mg, and without adjuvant.

The highest IC was observed in tests 3 and 4 at low concentration of EDMA (0.5% and 1%), highlighting the importance of the cross-linking agent for acrylamide polymerization, i.e., EDMA regulates the width of the matrix lattice. Moreover, IC and amount of EDMA were linearly correlated (Figure 5).

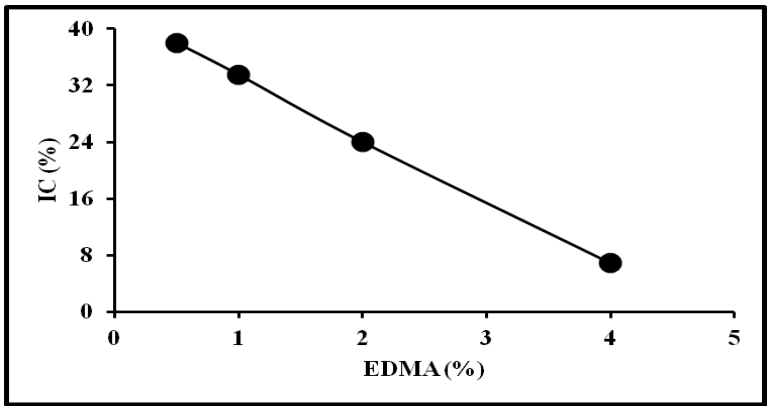

Figure 5: Correlation between immobilization coefficient (IC) and the amount of cross-linking agent (EDMA).

The standard minimum square linear regression equation is:

IC = 42.3 – 8.89.(EDMA) (r = - 0.9997) (Eq. 14)

Where: IC = immobilization coefficient (%); (EDMA) = amount of cross-linking agent (%).

The presence of polyethyleneglycol (PEG) as adjuvant did not improve immobilization yield although reducing polymerization time. Temperature at 20 ^{o}C sharply reduced polymerization time without increasing the IC. It is highlighted that both the temperature increase and the presence of adjuvant led to similar IC values (21.5% - 26.7%).

One of the objectives of immobilization is to use the immobilized catalyst in a bioreactor operated continuously.[12] Thereby, the stability of soluble and immobilized invertase performing sucrose hydrolysis inside the membrane bioreactor was evaluated during 100 h (Figure 6). The process rate (V_p) was expressed as μ moles of sucrose/h.mg of enzyme and calculated by equation:

$V_p = (\Delta A.F.V) \div 360.(\Delta t.E)$ (Eq. 15)

Where: ΔA = absorbance variation; F = inverse of the slope of the standard value of reducing sugars, obtained through Somogyi method (mg/Abs.mL); V = sample volume (mL); Δt = sample interval time (h); E= invertase amount (mg); 360 = mass conversion factor (g mol).

Sucrose conversion was calculated by equation:

$X = 100.[(S_o - S) \div S_o]$ (Eq. 16)

Where: X = fraction of sucrose consumed (%); S_o = initial sucrose concentration (mM); and S = sucrose concentration (mM).

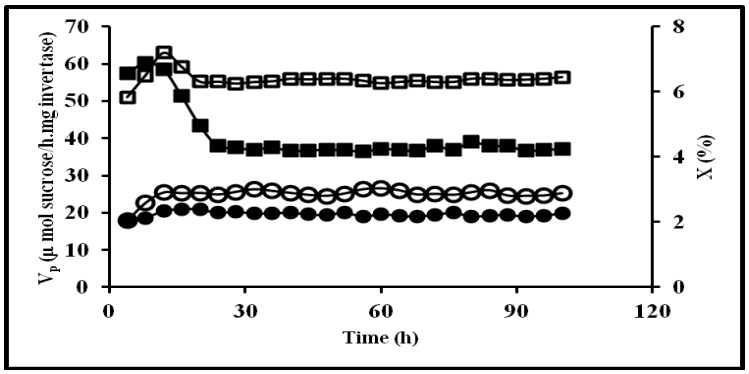

Figure 6: Sucrose hydrolysis (V_p) and sucrose conversion (X) of soluble (●;o) and immobilized (■;□) invertase in a membrane bioreactor (V_r = 100 mL; pressure = 0.3 bar; sucrose concentration = 40 mM) at 30 ^{o}C and pH 5.0 (50 mM acetate buffer). Ordinate values must be divided by 10^{-3} and 10^{-1} for soluble and immobilized invertase, respectively.

Figure 6 showed steady-state stability for both invertase forms. In addition, the process rate (V_p) for the immobilized form was lower than for the soluble form. Furthermore, sucrose conversion by immobilized invertase was higher than by soluble invertase.

Glucose Oxidase

Glucose oxidase (GO) is an oxidoreductase that converts β-D-glucose and oxygen into hydrogen peroxide and gluconic acid.[8] The role played by oxygen in catalysis is to recycle $FADH_2$ to FAD, allowing the enzyme to convert β-D-glucose into gluconic acid continuously. Thereby, the oxygen input into the bioreactor must be enough to ensure its saturation in the reaction medium. For complete conversion of 350-400 mM of β-D-glucose solution, the rate of O_2 bubbling was between 50-60 μL/s.

Kinetic parameters for soluble and immobilized GO were calculated by Hanes-Woolf method (Figure 7).

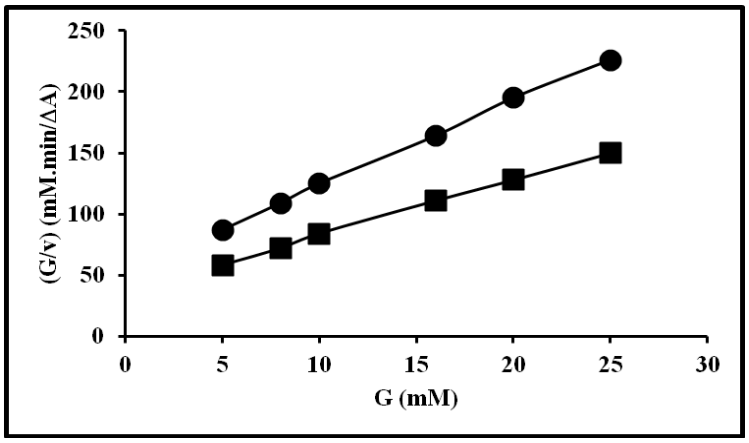

Figure 7: Hanes-Woolf plot for calculating the kinetic constants of soluble (■) and immobilized (●) glucose oxidase.

The standard minimum square linear regression equations are:

$(G/v)_s = 36.2 + 4.59.(G)$ (r = 0.9993) (Eq. 17)

$(G/v)_i = 53.5 + 6.96.(G)$ (r = 0.9995) (Eq. 18)

Where: (G) = glucose concentration (mM); $(G/v)_s$ and $(G/v)_i$ refer to soluble and immobilized glucose oxidase, respectively.

Thereby, the kinetic constant values were: $(V_{max})_s$ = 0.218 ΔA/min; K_{Ms} = 7.89 mM; $(V_{max})_i$ = 0.144 ΔA/min; and K_{Mi} = 7.69 mM.

It was observed for glucose oxidase that $(V_{max})_i < (V_{max})_s$, as normally occurs in immobilization procedures. However, $K_{Mi} \cong K_{Ms}$ is not commonly observed in immobilization techniques. Probably, the more complex catalysis mechanism presented by glucose oxidase – a Ping-Pong Bi-Bi mechanism.[16] – leads to random correlation between the Michaelis-Menten constants of both forms of GO. It is highlighted that in the present case, oxygen concentration was maintained at saturation level during catalysis, with the entire GO mechanism being dependent on one substrate (β-D-glucose).

Figure 8 shows the stability of immobilized GO, which was higher than that of soluble GO, indicating that the polyacrylamide hydrogel protects the enzyme against increasing temperatures, at least in the 30 ^{o}C – 50 ^{o}C interval.

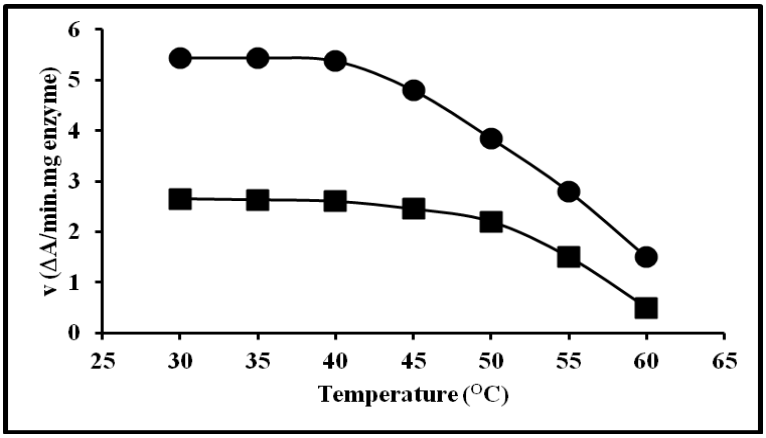

Figure 8: Effect of temperature on the stability of soluble (●) and immobilized (■) glucose oxidase. Ordinate values must be divided by 10^2 and 10^5 for soluble and immobilized GO, respectively.

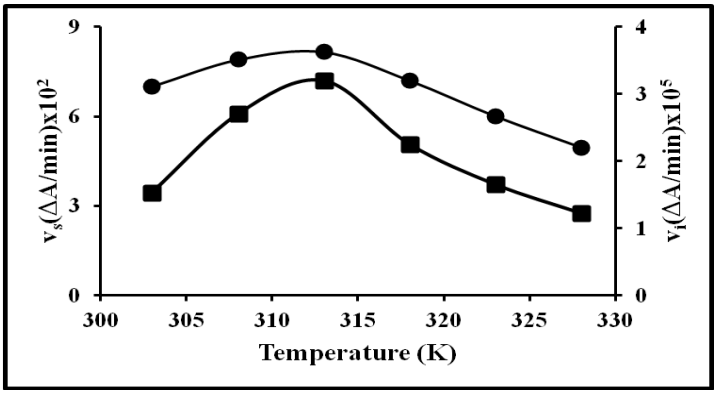

Figure 9: Effect of temperature on the activity of soluble (●) and immobilized (■) glucose oxidase.

Table 4: Thermodynamic parameters related to soluble and immobilized glucose oxidase. Temperature was set at 313K.

Enzyme	E_a (kJ/mol)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/mol.K)
Soluble	28.0	- 11.4	25.4	0.18
Immobilized	53.5	- 11.0	50.9	0.20

The highest activity for both forms of glucose oxidase (GO) occurred at 313K. Moreover, similar values of ΔG were observed (with difference of approximately 3.5%), although values of E_a and ΔH for immobilized GO were approximately 50% higher than that of soluble GO. Probably, the oxygen availability to GO and the diffusion barrier imposed by the hydrogel could explain the differences observed.

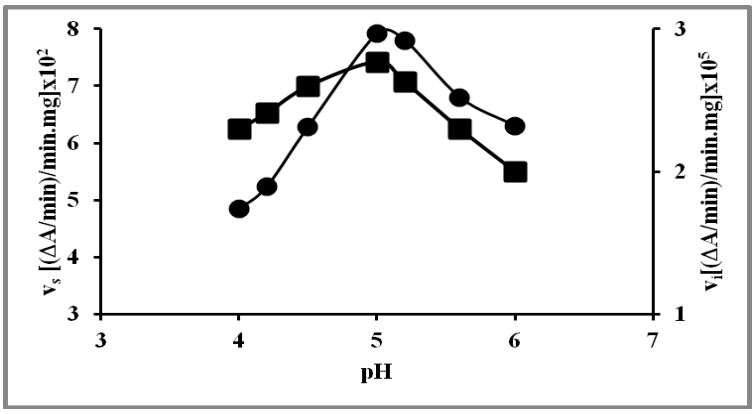

Figure 10: Effect of pH on the activity of soluble (●) and immobilized (■) glucose oxidase.

The highest activity occurred at pH 5.0 for both forms of GO. As the hydrogel has zero electric charge, the gradient environment phenomenon did not occur. This behavior was similar to that observed for soluble and immobilized invertase.

The study of glucose oxidase immobilization in polyacrylamide hydrogel considered the changing of the amount of cross-linking agent (EDMA: 0.5%, 1%, 2%, and 4%), enzyme amount (4.0, 8.0, 16.0 and 32.0 mg), and the type (PEG 1500 and PEG 6000) and amount of adjuvant (1% and 2%). Starter concentration (benzoin methyl ether = $1x10^{-4}$M) and temperature (4 ^oC) were maintained unchanged, except for tests 11 (10 ^oC) and 13 (20 ^oC). Data are shown in Table 5.

Table 5: Effect of procedure conditions on the immobilization coefficient (IC) of glucose oxidase in acrylamide hydrogel.

Test (n.)	Variation of parameter	Polymerization time (min)	IC (%)
1	*E = 8.0 mg	136	32.4
2	PEG6000: 2%	90	27.4
3	PEG6000: 1%	137	30.3
4	PEG1500: 1%	120	19.3
5	PEG1500: 2%	180	17.0
6	E = 16.0 mg	150	8.4
7	E = 32.0 mg	240	6.9
8	EDMA: 0.5%	211	24.3
9	EDMA: 1%	170	27.6
10	EDMA: 4%	110	19.7
11	T = 10 ^{o}C	90	32.0
12	E = 4.0 mg	90	10.2
13	T = 20 ^{o}C	90	29.3

*E = enzyme amount.

The highest IC, 32.4% and 32.0%, occurred in tests 1 and 11, respectively. The only difference between them was temperature, i.e., 10 ^{o}C (test 11) and 4 ^{o}C (test 1). The other conditions were E = 8.0 mg, EDMA: 2%, and polymerization starter ($1x10^{-4}$ M). Polymerization time, in turn, was markedly lower in test 11 than in test 1, leading to the conclusion that polymerization should be performed at 10 ^{o}C. Differently of invertase, the presence of PEG6000 at 1% led to an IC of 30.3% for GO (test 3).

Figures 11 and 12 show that polymerization time increses along with the amount of GO but decreases along with EDMA concentration (cross-linking agent) . As the interest is to obtain a high IC regardless of polymerization time, the amounts of EDMA and GO should be set at 2% and 8.0 mg, respectively.

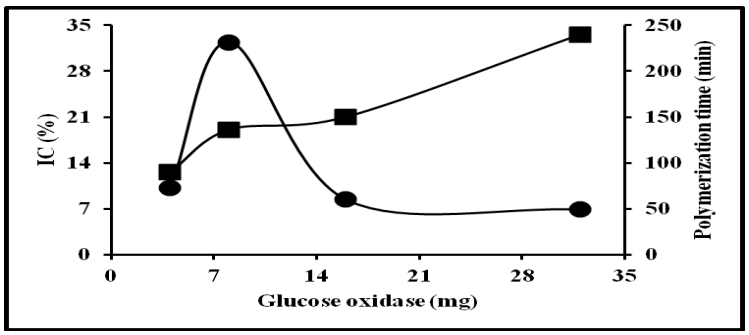

Figure 11: Effect of the amount of glucose oxidase on immobilization coefficient (●) and polymerization time (■).

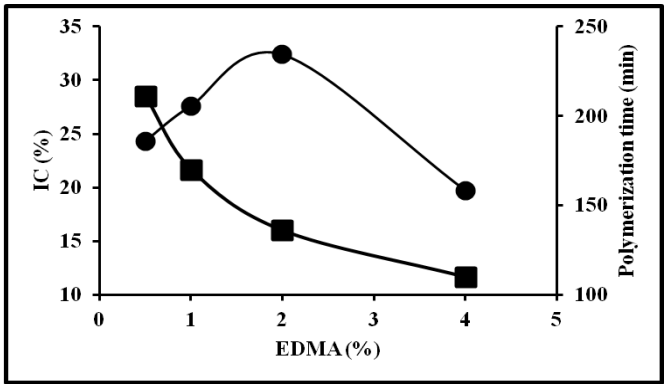

Figure 12: Effect of the concentration of cross-linking agent (EDMA) on the GO immobilization coefficient (●) and polymerization time (■).

The study on the stability of soluble and immobilized GO in a membrane bioreactor to oxidize glucose into gluconic acid was conducted similarly to the study on invertase (Figures 13 and 14). The process rate (V_p) was expressed as [(ΔA/min)/min.mg GO].

Glucose conversion was calculated by equation:

R = 100.(v/A) (Eq. 19)

Where: R = conversion yield (%); v = GO activity; A = total GO activity.

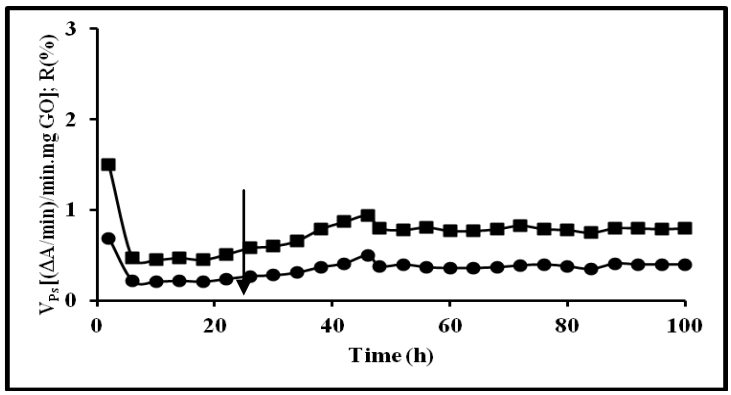

Figure 13: Glucose oxidation (V_{ps}) (●) and glucose conversion (R)(■) by soluble GO in a membrane bioreactor (V_r = 100 mL; pressure = 0.5/1.0 bar; glucose concentration = 40 mM) at 35 ^oC and pH 5.0 (50 mM acetate buffer). The arrow indicates the moment in which the bioreactor pressure was changed to 1.0 bar.

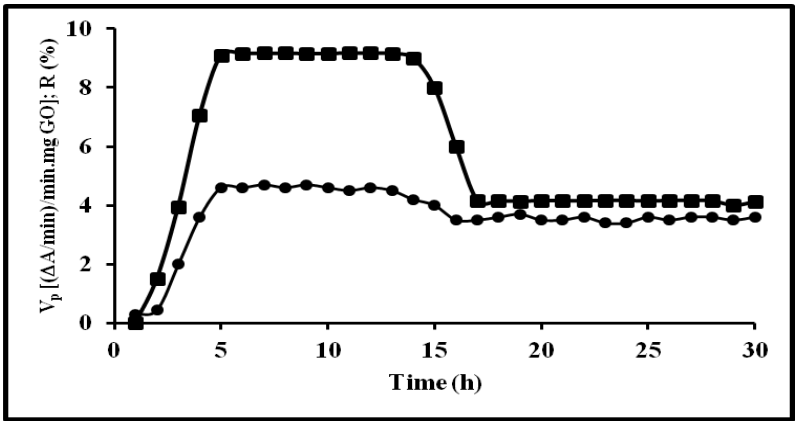

Figure 14: Glucose oxidation (V_p) (●) and glucose conversion (R)(■) by immobilized GO in a membrane bioreactor (V_r = 100 mL; pressure = 0.5 bar; glucose concentration = 40 mM, polymer amount = 300 mg at 35 ^{O}C and pH 5.0 (50 mM acetate buffer). Ordinate values must be divided by 10 and 0.10 for V_p and R, respectively.

Soluble GO activity and yield reached the steady-state after 47 h of continuous process and oxygen pressure of 1.0 bar (Figure 13). Immobilized GO also showed good performance, although sharply decreasing activity and yield after 15 h of continuous process occurred (Figure 14). This result can be explained by considering the GO mechanism as follows:[8]

[First] $G + \mathbf{FAD}\text{-GO-}Fe^{+2} \rightarrow GA + \mathbf{FADH_2}\text{-GO-}Fe^{+2}$

[Second] $\mathbf{FADH_2}\text{-GO-}Fe^{+2} + O_2 \rightarrow H_2O_2 + \mathbf{FAD}\text{-GO-}Fe^{+2}$

[Third] $\mathbf{FADH_2}\text{-GO-}Fe^{+2} + H_2O_2 \rightarrow H_2O + \mathbf{FADH_2}\text{-GO-}Fe^{+3} + ½\ O_2$

[Fourth] $\mathbf{FADH_2}\text{-GO-}Fe^{+3} + O_2 \rightarrow H_2O_2 + \mathbf{FAD}\text{-GO-}Fe^{+3}$

[Fifth] $G + \mathbf{FAD}\text{-GO-}Fe^{+3} \rightarrow GA + \mathbf{FADH_2}\text{-GO-}Fe^{+3}$

Where: G= glucose; GA = gluconic acid.

As observed, the ferrous ion is oxidized to ferric ion due to the presence of hydrogen peroxide leading to a less active GO on glucose oxidation. Such situation reached a maximum after 15 h of continuous process. This effect could be avoided if catalase was added in the bioreactor. Such effect is more pronounced on immobilized GO due to hindrance by diffusion imposed to the molecular movement (substrates and products) through the hydrogel matrix.

CONCLUSION

The data obtained led to the conclusion that entrapment in polyacrylamide hydrogel must be improved regarding enzyme amount, concentration of cross-linking agents and, in the case of glucose oxidase immobilization, polymerization temperature. The immobilization coefficient (IC) obtained either for invertase or glucose oxidase ranged between 30% and 36%. According to the literature, ICs over 50% are quite common for this immobilization type. An interesting result was the linear correlation between IC and concentration of cross-linking agent, as the width of matrix-cells can be adjusted to increase the retention capability of the hydrogel. All enzyme forms were stable when maintained for at least 30 h in a membrane bioreactor operated in continuous mode.

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