WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.632
Volume 10, Issue 3, 232-251 Research Article ISSN 2278 – 4357

IMMOBILIZATION ON CHITIN AND POLYETHYLENE OF INVERTASE OBTAINED FROM YEAST GROWN IN MOLASSES BY FED-BATCH PROCESS

Michele Vitolo*

Department of Pharmaceutical and Biochemistry Technology, School of Pharmaceutical Sciences, University of São Paulo, Brazil.

Article Received on
11 January 2021,

Revised on 01 Feb. 2021,
Accepted on 21 Feb. 2021

DOI: 10.20959/wjpps20213-18531

***Corresponding Author**
Michele Vitolo
Department of Pharmaceutical and Biochemistry Technology, School of Pharmaceutical Sciences, University of São Paulo, Brazil.

ABSTRACT

Saccharomyces cerevisiae is largely used in industry and in biochemical studies. The main product extracted from this yeast is invertase, which has many applications, for instance in syrup production in either soluble or immobilized form. This study dealt with invertase production from *S. cerevisiae* grown in blackstrap molasses by fed-batch fermentation. Fermentation parameters as specific rate of cell growth (μ), substrate consumption, generation time, and invertase activity of intact yeast cells were determined. The invertase present in cells obtained by exponential fed-batch culture was extracted and purified. The purified invertase was immobilized on chitin (adsorption plus cross-binding with glutaraldehyde) and polyethylene (covalent binding). The kinetic (K_M and V_{max}) and thermodynamic (E_a, ΔG, ΔH, and ΔS) constants of all forms of invertase were calculated.

KEYWORDS: Invertase, chitin, polyethylene, yeast, molasses, fed-batch.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* belongs to the class ***Ascomycetes***, sub-class ***Hemiascomycetidae***, and ***Endomycetales*** group. This strain grows preferentially by budding (asexual cell division), but in some special conditions the cell forms an ascus in which the daughter cells undergo meiosis originating positive (+) and negative (-) haploid descendants. These descendants multiply by budding and, after a lapse of time, two haploid cells (+/-) fuse, restoring the diploid phase (Figure 1). The extensive literature on *S. cerevisiae*

highlights it as an important microorganism.[1] It is easily isolated, maintained in simple and low cost medium (water, sugars such as glucose and sucrose, and common salts such as ammonium sulphate and sodium phosphate), and grows in industrial residues (e.g., blackstrap molasses and corn steep liquor). This yeast can be either a transformation agent in industrial processes (baking, ethanol production, and alcoholic beverages such as beer, wine, and liquors) or a source of several products (enzymes, yeast extract for microbiological use, nucleic acids, and source of vitamin B for animal feed formulae or compressed into tablet form for pharmaceutical use). Under non growing condition, the yeast *per se* can be used in sucrose hydrolysis for invert syrup production. In this case, it acts as an inert carrier for periplasmic cell wall-associated invertase.[2] It is also used as a microbial model in modern biotechnology.[3]

Although S. cerevisiae per se has great commercial relevance, invertase (β-D-fructofuranosidase; E.C.3.2.1.26) is by far the most famous derivative. Invertase has been used in confectionary industry, in inverted sugar production, in enzyme electrodes for sucrose determination, and as a medicine (associated or not with other enzymes) for antimicrobial and antioxidant purposes.[4,5] Furthermore, invertase in the yeast cell wall makes the cell a natural carrier for invertase immobilization. However, using whole intact yeast cell has two disadvantages, i.e., the amorphous form of wet cells – inadequate for using in plug flow bioreactor because it is submitted to internal pressure leading to high bed compaction – and the possibility of the products (glucose and fructose) being adsorbed and metabolized by the yeast. It is thus advisable to extract the invertase from the cell and, after purification, immobilizing it on inert carriers by entrapment, adsorption, or covalent chemical binding.[6,7]

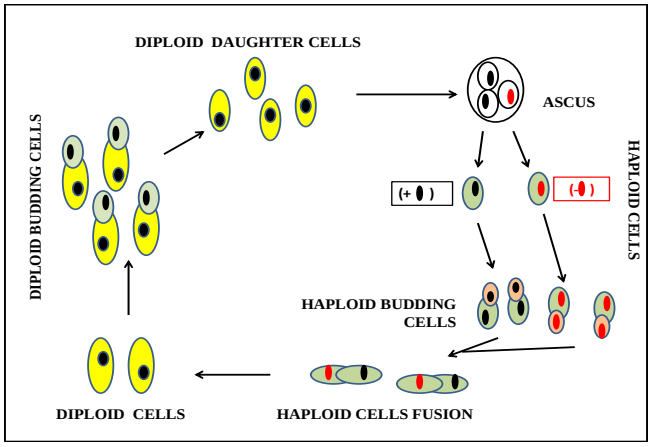

Figure 1: Diagram of the *Saccharomyces cerevisiae* life cycle. Haploid mates: black (positive) and red (negative).

Fermentation is an industrial process involving cell growth in association with product(s) formation. It can be conducted by batch, continuous, or fed-batch mode.[8] The fed-batch mode is chosen when an enzyme, as invertase, is subjected to catabolite repression in the presence of high amounts of substrate and/or product. This choice is because the substrate can be added parsimoniously into the fermenter so as to avoid reaching an inhibitory concentration. Thus the accumulation of the inhibiting product inside the fermenter can be controlled.[9]

This study dealt with invertase production from *S. cerevisiae* grown in blackstrap molasses by fed-batch fermentation. The cells obtained through the best fermentation condition were submitted to an alkaline extraction followed by a sequence of unit operations according to a downstream protocol. The purified invertase was immobilized on chitin (adsorption + cross binding with glutaraldehyde) and polyethylene (covalent binding).

MATERIALS AND METHODS

Material

The culture of *Saccharomyces cerevisiae* used was isolated and purified from compressed yeast of Fleischmann-Royal Inc. (São Paulo, Brazil). Culture media and blackstrap molasses were purchased from DIFCO (Leeuwarden, The Netherlands) and Copersucar (São Paulo, Brazil), respectively. All analytical P.A. grade reagents were purchased from traditional suppliers.

Methods

Fermentation

The chemical treatment of blackstrap molasses and the inoculum preparation followed literature procedures.[1]

A typical fed-batch fermentation test was performed by introducing 1.55 L of treated molasses (with a reducing sugar concentration of 5.0 g/L; 2.4 g/L of $Na_2HPO_4.12H_2O$; 0.075 g/L of $MgSO_4.7H_2O$; and 5.1 g/L of $(NH_4)_2SO_4$) and 0.45 L of inoculum into a 5-L fermenter. The suspension was left for 3 h in batch mode. Then, a sucrose solution (30 g/L) was added step by step to the fermenter, being the feeding rate calculated from one of the equations shown in Table 1. The volume inside the fermenter increased from 2 L to 3 L. Then, the suspension was left batchwise until complete sugar exhaustion. The fermentation

conditions maintained constant were agitation (500 min^{-1}), air inlet (1 L/L.min), inoculum age (22 h), pH (4.5), temperature (35 ^{o}C), and whole amount of sucrose added (30 g).

In the case of pulse feeding, 2.05 L of molasses and 0.45 L of inoculum were added to the fermenter, and the suspension was left fermenting batchwise for 3 h or 4 h. Then, 0.5 L of sucrose solution (30 g/L) was added and fermentation continued until complete sugar exhaustion. When the pulse was divided in two portions of 0.25 L, each portion was added 3h and 6 h after the start of fermentation. Also in these cases, the suspension was left fermenting until exhaustion of reducing sugars. Table 2 indicates the conditions of all tests.

Table 1: Equations for sucrose solution feeding in a fed-batch fermenter.[10]

t_f (h)	**Linearly Decreasing Mode [$(V - V_o) = F_o.t - k.(t^2/2)$]**
2	$(V - V_o) = t - (t^2/4)$
4	$(V - V_o) = (t/2) - (t^2/16)$
t_f (h)	**Linearly Increasing Mode [$(V - V_o) = F_o.t + k.(t^2/2)$]**
2	$(V - V_o) = t^2/4$
4	$(V - V_o) = t^2/16$
t_f (h)	**Exponentially Decreasing Mode [$(V - V_o) = - (F_o/k).(e^{-kt} - 1)$]**
2	$(V - V_o) = \{1/[1 - (2.72)^{-1.2}].[1 - (2.72)^{-0.6t}]\}$
4	$(V - V_o) = \{1/[1 - (2.72)^{-2.4}].[1 - (2.72)^{-0.6t}]\}$
t_f (h)	**Exponentially Increasing Mode [$(V - V_o) = (F_o/k).(e^{kt} - 1)$]**
2	$(V - V_o) = \{[1/(2.72)^{1.2} - 1].[(2.72)^{0.6t} - 1]\}$
4	$(V - V_o) = \{[1/(2.72)^{2.4} - 1].[(2.72)^{0.6t} - 1]\}$

t = fermentation time (h); t_f = feeding time (h); V = volume of medium (L); V_o = initial volume of medium (L); F_o = feeding rate (L/h); k = constant of addition (L/h^2) or (h^{-1}).

Table 2: Test conditions for all tests, identified by the numbers in parenthesis.

Addition	t_f			
Mode	**2 h**	**3 h**	**4 h**	**6 h**
One pulse I		(1)		
One pulse II			(2)	
Double pulse		(3)		(3)
Linearly decreasing	(4)		(5)	
Linearly increasing	(6)		(7)	
Exponentially decreasing	(8)		(9)	
Exponentially increasing	(10)		(11)	

Each culture was followed by taking samples (20 mL) at one hour intervals. Ten mL of the sample were filtered through a tarred 0.45-μm membrane, and ten mL were centrifuged (3,000x*g*). The filtrate was used for determining the concentration of reducing sugars, whereas the cell cake deposited on the membrane was rinsed with two portions of 10 mL of

distilled water, followed by drying the cake and the membrane in a stove at 105 ^{o}C until constant weight (dry cell mass). The cake resulted from the centrifugation was collected and rinsed twice with 10 mL of distilled water. Then, the cake was suspended in 25 mL of distilled water and left at 4 ^{o}C until use (determination of yeast invertase activity). The 20 mL of sample volume was compensated by adding 20 mL of sterile deionized water to the fermenter.

Invertase production

For obtaining invertase, firstly, 3.0 g of cells and 200 mL of 0.154 M $NaHCO_3$ were introduced into a 500-mL Erlenmeyer flask. Then, the flask was left stirring (200 rpm) for 24 h, followed by centrifugation at 3,000x*g*/30 min, being the supernatant I collected. To the supernatant I was added sodium picrate solution (0.13 M) at a volumetric ratio of 1:0.35, and the mixture was left to rest for 3 h. The suspension was centrifuged at 3,000x*g*/30 min and the precipitate was discharged. To the supernatant II was added acetone at a ratio of 3:1, and the mixture was left to rest for 2 h. The precipitate was separated by centrifugation (3,000x*g*/1 h) and dried under low pressure (0.2 bar) at 30 ^{o}C for 5 h. The invertase was dissolved in 0.05 M phosphate buffer (pH 7.0), followed by filtration through a DEAE-cellulose column (100-mesh), previously equilibrated with the same buffer. Invertase was eluted with 0.1 M TRIS-HCl buffer (pH 7.0) containing 0.5 M of NaCl. Finally, the eluant was collected and dialyzed against 50 volumes of 0.1 M TRIS-HCl (pH 7.0) for 16 h at 4 ^{o}C. This invertase solution was used for immobilization on chitin and polyethylene.

Invertase Immobilization

Chitin

Chitin was prepared as previously described.[11]

Firstly, 20 mL of deionized water, 30 mg of chitin, invertase solution (1 mg/mL), and glutaraldehyde (1 g/L) were introduced into a 50-mL beaker. The mixture was maintained at 30 ^{o}C, being gently stirred for one minute at each 15 min for 1 h. Then, the suspension was left to rest for 24 h at 4 ^{o}C, followed by centrifugation (1,000x*g*/10 min). The precipitate (chitin-invertase complex) was collected and stored at 4 ^{o}C until use. The protein content in the supernatant was determined.

Polyethylene

Polyethylene (density = 0.91) was functionalized for immobilization as previously described.[12]

Firstly, 50 mL of buffered solution of invertase (1 mg/mL) and 1 g of functionalized polyethylene were introduced into a 250-mL beaker. The suspension was left stirring at 250 rpm for 24 h at 2 ^{o}C. Then, the suspension was filtered and the complex polyethylene-invertase collected and stored at 4 ^{o}C until use. The protein content in the filtrate was determined.

Invertase Activity Determinations (Standard Tests)

Immobilized invertase

Fifty milligrams of immobilized invertase and 100 mL of 0.01 M acetate buffer (pH 4.6) were poured into a 250-mL beaker coupled with a magnetic stirrer and left stirring at 200 rpm for 10 min at 37 ^{o}C. Then, 50 mL of 0.2 M sucrose solution were added and the reaction was followed for 1 h. At each 15 min, a 1 mL sample was taken for determining the reducing sugars (RS) formed. Immobilized invertase activity (IIA) was calculated from the slope of RS = f(t). One unit (U_{IIA}) was defined as the amount of IIA catalyzing the formation of 1 mg of RS per minute at pH 4.6 and 37 ^{o}C. Specific IIA was defined as U_{IIA}/mg of protein.

Soluble invertase

The procedure was similar to that of immobilized invertase, except for overall reaction time (10 min) and sampling interval (1 min). One unit (U_{SI}) was defined as the amount of soluble invertase (SI) catalyzing the formation of 1 mg of RS per minute at pH 4.6 and 37 ^{o}C. Specific SI was defined as U_{SI}/mg of protein.

Characterization of Immobilized Invertase

Effect of temperature on invertase activity

Standard tests were carried out at a temperature interval of 30 ^{o}C – 65 ^{o}C. Thermodynamic parameters were calculated through conventional thermodynamic relations and the Arrhenius method.[13]

Effect of temperature on invertase stability

The IIA suspension and SI solution were incubated for 30 min at temperatures between 30 ^{o}C and 70 ^{o}C. Then, the residual activity was determined under standard test conditions.

Effect of pH on invertase activity

Standard tests were carried out at different pH in 0.2 M buffers as follows: acetate buffer (3.8, 4.0, 4.6, 5.0, and 5.6), succinate buffer (6.0 and 6.4), and cacodylate buffer (7.0).[10]

Effect of pH on invertase stability

The IIA suspension and SI solution were incubated for 15 min in 0.2 M buffers: acetate (pH: 3.8, 4.0, 4.6, 5.0, and 5.6), succinate (pH: 6.0 and 6.4), and cacodylate (7.0). Then, the residual activity was determined under standard test conditions.

Kinetics constant calculation

Standard tests were conducted at sucrose concentrations of 4.5, 5.0, 5.5, 6.0, 8.0, 10.0, 15.0, 20.0, 30.0, and 45.0 mM. The constants (K_M and V_{max}) were calculated by the Hanes-Woolf plot method.[10]

Analytical Procedures

Cell mass determination

Dry cell mass was determined as previously described.[1] Cell concentration was expressed as g/L.

Soluble protein determination

Soluble protein was determined using the Bradford reactive.[10] The standard curve was made with bovine albumin solution (0.4 mg/mL), in which the amount of protein varied between 40 μg and 200 μg. The minimal square linear regression equation is represented by:

$Y = 2.2x10^{-3}x - 0.002$ (r = 0.998) (Eq. 1)

Where: Y = absorbance; x = amount of protein (μg).

Reducing sugars determination

The reducing sugars (RS) concentration, expressed as g/L, was determined by using the Somogyi-Nelson method, with color intensity read at λ = 540 nm.[1,10] The standard curve was made with glucose solutions ranging from 40 μg to 200 μg. The minimal square linear regression equation is represented by:

$Y_{RS} = 2.1x_1 + 0.011$ (r = 0.995) (Eq. 2)

Where: Y_{RS} = absorbance; x_1 = amount of glucose (μg).

Invertase activity of intact yeast cells

Initially, 2.5 mL of 0.2 M sucrose solution were added to a 25 mL-Folin-Wu tube, followed by immersion in water bath at 37 ^{O}C for 10 min. Then, 0.5 mL of cell suspension was added and the mixture was left reacting at 37 ^{O}C for 1, 2, or 3 minutes. The reaction was stopped by adding 1.0 mL of alkaline-cupric solution, followed by immersion in boiling water for 10

min. After that, the procedure followed as previously described.[1] The invertase activity of intact yeast was calculated by using the equation:

$v = (2fV_I/m.t).[(y – y_o – a)/b]$ (Eq. 3)

Where: v = specific invertase activity (g RS/g cell.min); f = dilution factor; m = dry cell mass (g); t = reaction time (min); V_I = volume of the sample (mL); y = absorbance read at 540 nm; y_o = initial absorbance read at 540 nm; a = slope of the standard curve; b = linear coefficient of the standard curve.

One yeast invertase unit (U_Y) was defined as the amount of enzyme catalyzing the formation of 1 g of reducing sugars per min at pH 4.6 and 37°C. Invertase production (v') was expressed as U_Y/L. Specific invertase activity was expressed as U_Y/g cell.

RESULTS AND DISCUSSION

Exploratory tests, in which the substrate solution was added in one or two portions (pulse feeding), were performed to observe if a sucrose concentration of 30 g/L was sufficient to cause a measurable response of fermentation in terms of cell and invertase formation. The main results regarding the cell (X) and reducing sugars (RS) concentrations and the invertase activity of intact yeast cells (v) were shown in Table 3.

Table 3: Fermentation tests in which the sucrose solution was added by pulse.

Test (n.)	Parameter	t (h)									
		0	1	2	3	4	5	6	7	8	10
1	X (g/L)	0.60	0.71	0.84	1.3	1.6	2.2	2.9	4.0	4.6	5.0
	RS (g/L)	7.4	6.1	5.0	3.1	28	24	18	8.9	1.9	0.05
	v (U_Y/g cell)	0.48	0.50	0.41	0.33	0.21	0.13	0.11	0.082	0.55	0.62
2	X (g/L)	0.60	0.78	0.95	1.3	1.9	2.3	3.1	4.1	5.2	5.9
	RS (g/L)	32	30	29	26	23	37	29	16	8.9	1.1
	v (U_Y/g cell)	0.42	0.40	0.29	0.20	0.14	0.10	0.098	0.073	0.25	0.65
3	X (g/L)	0.57	0.62	0.85	1.2	1.7	2.4	3.0	3.6	4.2	4.6
	RS (g/L)	7.2	6.2	4.8	2.6	12	6.8	1.1	4.2	0.57	0.07
	v (U_Y/g cell)	0.49	0.52	0.40	0.35	0.24	0.16	0.73	0.52	0.73	0.78

Table 3 shows that, under the experimental conditions, fermentation responded sharply to the variation of sucrose concentration added, mainly in the double pulse test (Figure 2). As can be seen from Figure 2, invertase activity decreased at the peaks of reducing sugars concentration (at 4 and 7 h). This is a clear demonstration that invertase biosynthesis in yeast is subjected to catabolite repression.

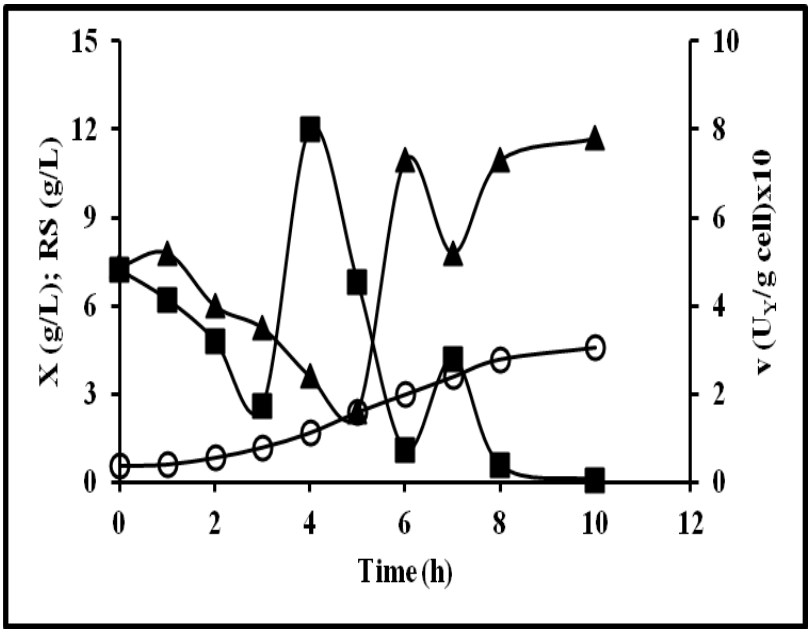

Figure 2: Variation of cell (O) and reducing sugars (■) concentrations, and invertase activity of intact yeast cell (▲) against time for Test 3.

Table 4 shows that, between 4 h and 10 h, specific invertase activity increased as fermentation continued. In all cases, the reducing sugars concentration was below 3.0 g/L at t = 4 h, an up-limit concentration under which the intensity of catabolite repression is reduced. Moreover, tests 7, 10, and 11, in which the reducing sugars concentration was below 0.5 g/L at t = 4 h, led to cells with specific invertase activity above 1.5 U_Y/g cell. For instance, Figure 3 shows the variation of X, RS, and v against fermentation time for Test 11. Although specific invertase activity increased from t = 4 h, in all cases the final cell invertase activity never surpassed 2.0 U_Y/g cell. One possible explanation is that the culture medium was impoverished of basic nutrients (sugars, magnesium, phosphorous, and nitrogenous salts) at the end of fermentation.

The data shown in Table 4 indicate high invertase activity (1.8 – 1.9 U_Y/g cell) for Tests 7 (linearly increasing; t_f = 4 h), 10 (exponentially increasing; t_f = 2 h), and 11 (exponentially increasing; t_f = 4 h). As the actual source of invertase is the yeast cell, it was interesting to calculate invertase productivity (v') at the end of fermentation. Clearly, tests operated in exponentially increasing mode led to the highest productivities (5.13-5.22 U_Y/L) (Table 4). This result corroborates the fact that in exponentially increasing fed-batch mode the sucrose is added more slowly than in the other fed-batch modes.[10] Thereby, invertase catabolite repression is minimized.

The specific cell growth rate (μ) is a derivative parameter of fermentation that can be calculated by Equation 4 or 5. [2][8][14] The generation time (t_g), i.e., the time during which one yeast cell divides in two daughter cells (Figure 1) can be calculated by Equation 6.

$\mu = (1/X).(dX/dt)$ (Eq. 4)

$Ln\ X = Ln\ X_o + \mu t$ (Eq. 5)

$t_g = (Ln\ 2) \div \mu$ (Eq. 6)

Where: X = cell concentration (g/L); X_o = initial cell concentration (g/L).

The specific growth rate (μ) can be calculated from X = f(t) plot (Figure 4) and by applying Equations 4 and 5. The calculation of μ by Equation 4 implies evaluating the derivative (dX/dt) at each point of the curve X=f(t), meanwhile Equation 5 allows to calculate μ by the slope of the Ln X = f(t) curve (Figure 4). [2][14]

Table 4: Data of fed-batch fermentation tests.

Test (n.)	Parameters	t (h)					
		0	**2**	**4**	**6**	**8**	**10**
4	**X (g/L)**	0.70	1.1	1.7	2.5	2.6	2.8
	RS (g/L)	5.5	3.3	3.1	0.96	0.07	0.02
	v (U_Y/g cell)	0.38	0.28	0.98	0.70	0.99	1.1
	v' (U_Y/L)	**3.08**					
	t_g (h)	**3.6**					
5	**X (g/L)**	0.78	1.1	1.8	2.4	2.8	2.8
	RS (g/L)	5.7	4.1	3.9	0.95	0.11	0.05
	v (U_Y/g cell)	0.39	0.32	0.78	0.82	1.4	1.4
	v' (U_Y/L)	**3.92**					
	t_g (h)	**3.9**					
6	**X (g/L)**	0.70	1.1	1.9	2.2	2.7	2.7
	RS (g/L)	5.7	3.5	0.45	3.0	0.28	0.06
	v (U_Y/g cell)	0.44	0.37	0.76	0.67	1.2	1.2
	v' (U_Y/L)	**3.24**					
	t_g (h)	**3.8**					
7	**X (g/L)**	0.75	1.1	2.0	2.7	2.7	2.7
	RS (g/L)	5.1	2.5	0.36	0.22	0.07	0.02
	v (U_Y/g cell)	0.45	0.39	0.90	1.1	1.4	1.8
	v' (U_Y/L)	**4.86**					
	t_g (h)	**4.9**					
8	**X (g/L)**	0.85	1.4	1.7	3.0	2.9	3.0
	RS (g/L)	5.7	2.1	4.3	0.49	0.18	0.03
	v (U_Y/g cell)	0.49	0.47	1.2	1.1	1.1	1.1
	v' (U_Y/L)	**3.30**					
	t_g (h)	**4.8**					

9	**X (g/L)**	0.73	1.3	1.7	2.4	2.6	2.7
	RS (g/L)	5.4	2.1	2.7	0.26	0.09	0.02
	v (U_Y/g cell)	0.64	0.45	0.95	1.2	1.3	1.4
	v' (U_Y/L)	**3.78**					
	t_g (h)	**4.3**					
10	**X (g/L)**	0.68	1.1	1.7	2.6	2.8	2.9
	RS (g/L)	5.2	3.0	0.84	0.64	0.40	0.07
	v (U_Y/g cell)	0.62	0.42	0.85	1.2	1.4	1.8
	v' (U_Y/L)	**5.22**					
	t_g (h)	**4.0**					
11	**X (g/L)**	0.78	1.2	1.8	2.8	2.8	2.7
	RS (g/L)	5.2	2.2	0.11	0.18	0.08	0.02
	v (U_Y/g cell)	0.43	0.40	1.0	1.2	1.5	1.9
	v' (U_Y/L)	**5.13**					
	t_g (h)	**5.8**					

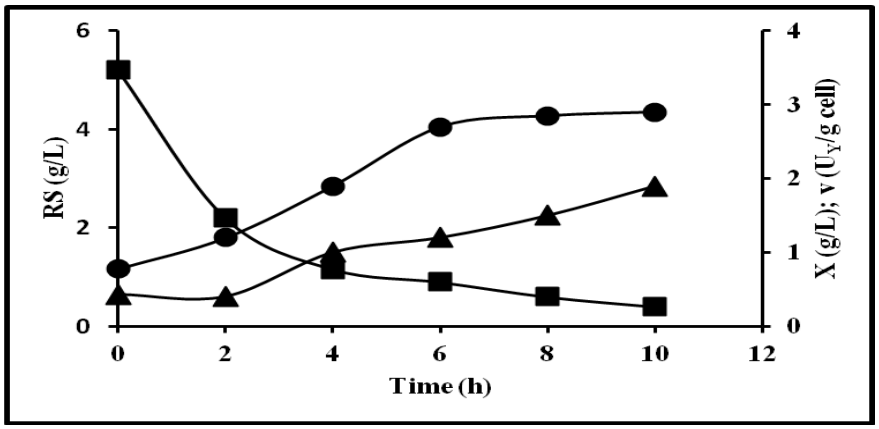

Figure 3: Variation of cell (X; ●) and reducing sugars (RS; ■) concentrations, and specific invertase activity (v; ▲) against fermentation time for Test 11.

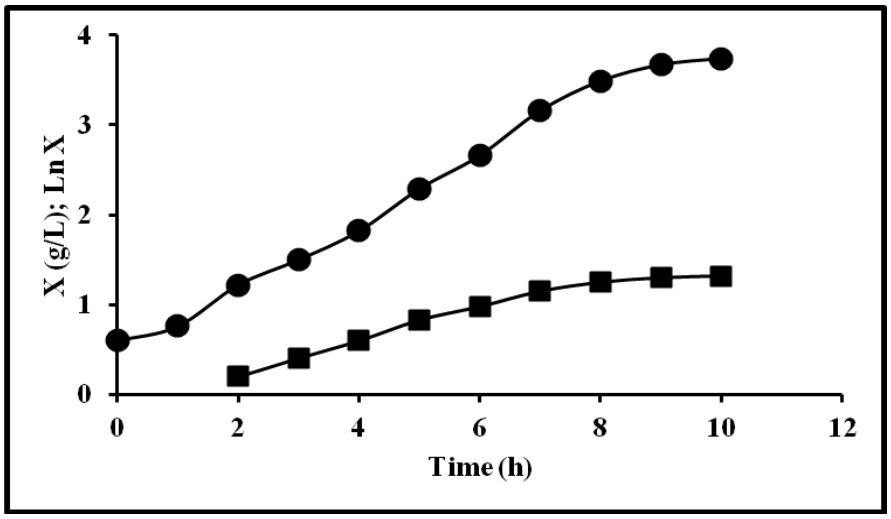

Figure 4: Variation of cell concentration (●) and the logarithm of X (■) versus time for Test 10.

Considering the time interval of 3 – 8 h, which corresponds to the exponential growth phase (see the X=f(t) curve), we can see from Figure 4 that Ln X varied linearly with time. Therefore, the minimum square linear regression equation is:

Ln X = - 0.078 + 0.172.t (r = 0.993) (Eq. 7)

Where: t = time (h).

The slope of Equation 7 corresponds to the specific growth rate ($\mu = 0.172\ h^{-1}$). By applying Equation 6, the generation time (t_g) for Test 10 was 4.0 h. Generation times for other tests are presented in Table 4. Noteworthy, the t_g varied as a function of the way sucrose was added to the fermenter.

Moreover, to calculate μ at each point of the X=f(t) curve, the method developed by Le Duy and Jazik must be used. [14] Taking the cell concentration values shown in Table 4 for Tests 7, 10, and 11, the μ calculated at one hour intervals are presented in Table 5.

Table 5: Values of the specific growth rate (μ) and specific invertase activity of yeast (v) versus time for Tests 7, 10, and 11.

Test (n.)		t (h)									
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
7	****μ**	0.14	0.26	0.29	0.20	0.17	0.13	0.11	0.09	0.06	0.02
	***v**	0.64	0.44	0.39	1.18	1.27	1.30	1.31	1.31	1.32	1.32
	v/μ	4.6	1.7	1.3	5.9	7.5	10	11.9	14.6	22	66
10	**μ**	0.03	0.26	0.35	0.25	0.19	0.16	0.13	0.05	-	-
	v	0.62	0.60	0.42	1.11	1.22	0.80	0.90	0.99	1.02	1.02
	v/μ	20.6	2.3	1.2	4.4	6.4	5.0	6.7	19.7	-	-
11	**μ**	0.05	0.19	0.32	0.25	0.15	0.12	0.10	0.07	0.04	-
	v	0.43	0.52	0.41	1.05	1.28	1.24	1.43	1.18	1.17	1.23
	v/μ	8.6	2.7	1.3	4.2	8.5	10.3	14.3	16.9	29.3	-

* Yeast specific invertase activity (U_Y/g cell);**μ expressed as h^{-1}.

According to the literature, when $\mu \leq 0.10$ h-1, cell budding decreases and individual cells predominate in the culture medium. Such situation has been achieved in the present study after 6 h of fermentation (Table 5). During this process, physiological modifications related to gene repression and enzyme inactivation are likely to occur.[15,16,17,18] In other words, the cell cycle is interrupted at the G1 phase, in which individual cells predominate and several events occurs such as interruption of DNA synthesis, accumulation of trehalose, and depletion of cAMP.[19] Budding interruption could result from an imbalance between the carbon source and key nutrients – mainly magnesium, nitrogenous, and phosphorous salts.

This situation could be directed to increase invertase formation and cell multiplication if after 7 h of process a new charge of salts was added to the fermenter and the sucrose feeding re-started. That is, fermentation would be carried out by successive fed-batches. This approach could be operated by using a high volume fermenter.

We can see from Table 5 that, after t = 2 h, μ decreased as v increased, showing an apparent uncoupling between growth and invertase biosynthesis. However, it is noteworthy that enzyme formation is subjected to catabolite repression, which depends on the reducing sugars concentration in the medium (Figure 3). Figure 3 shows that the yeast grows continuously along with fermentation, and invertase formation increases only after 2-3 h after the beginning of fermentation. After this point, yeast growing and invertase formation are, in fact, coupled events. Moreover, the ratio v/μ was always above 1.0, a clear indication that the amount of invertase formed was not a limiting factor to the availability of reducing sugars to the yeast during fermentation.

Cells harvested from Test 10 – in which the highest invertase production was obtained (Table 4) – were used for invertase extraction. The downstream protocol for invertase purification and the parameters associated to each phase are shown in Table 6, which demonstrates that the final purification factor obtained in the dialyzed was 6.6.

Table 6: Downstream phases for invertase purification.

Phase (name)	Protein (mg/mL)	Volume (mL)	[a]AI (U_{SI}/mL)	[b]AIT (U_{SI})	[c]AIE (U_{SI}/mg protein)	[d]PF
Autolyzed	6.5	45	0.53	24	0.082	-
Supernatant I	4.4	48	0.44	21	0.100	1.22
Supernatant II[e]	1.3	50	0.34	17	0.262	3.20
Eluate	1.0	45	0.46	21	0.46	5.61
Dialyzed	1.0	20	0.54	11	0.54	6.6

[a]AI = Invertase activity; [b]AIT = Total invertase activity; [c]AIE = specific invertase activity; [d]PF = purification factor; [e]Supernatant resulted from the resuspension in deionized water of invertase precipitated with acetone from supernatant I.

The dialyzed was concentrated under low pressure (0.2 bar) at 32 ^{o}C, followed by immobilization of invertase on chitin or polyethylene as described above.

The formation of reducing sugars as a function of reaction time for all forms of invertase was presented in Figures 5, 6, and 7. The minimum square linear regression equations were:

$Y_{SI} = 1.42t + 0.05$ (r = 0.9996) (Eq. 8)

$Y_{IPI} = 1.14t + 0.612$ (r = 0.9992) (Eq. 9)

$Y_{ICI} = 0.326t + 0.016$ (r = 0.9997) (Eq. 10)

Where: t = reaction time (min); Y_{SI}, Y_{IPI}, and Y_{ICI} refer to the reducing sugars (mg) formed, respectively, by soluble invertase and invertase immobilized on polyethylene and chitin.

The activities for SI, IPI, and ICI were 1.42 U_{SI}/mg protein, 1.14 U_{IPI}/mg protein, and 0.326 U_{ICI}/mg protein, respectively. Immobilization on polyethylene and chitin led respectively to activities 19.7% and 77% lower than that of the soluble form. This is a common result between the immobilized and soluble forms since immobilization limits the enzyme-substrate interaction due to effects such as low diffusion, steric and conformational enzyme change, partition between the microenvironment and the bulk of solution of charged ions and/or molecules (H_3O^+, for instance) etc.[7] The difference in activity between IPI and ICI could be due to the location of invertase molecules into the support. In the case of IPI, invertase molecules are linked to chemical groups located on the polyethylene surface, becoming available to interact with sucrose. In ICI, the invertase is entrapped in a web-like matrix constituted by chitin cross-linked with glutaraldehyde, which restrains diffusion in sucrose-invertase interactions.

At this point it is important to ask: why to immobilize invertase? The reasons pending to immobilization are basically two, i.e., reutilization of the enzyme and use of continuous reactors. Another question refers to the type of support to be used, that is, polyethylene or chitin? The response to these questions must consider the overall cost of enzymatic hydrolysis of sucrose either by soluble or insoluble invertase forms. As the hydrolytic process at industry scale involves tons of sucrose and, consequently, huge amounts of soluble invertase, so using the immobilized invertase could sound economically favorable. Although the supports led to a significant difference on invertase activity, the choice between them can also be based on their production costs insofar as the preparation of polyethylene is more expensive than that of chitin. Chitin is obtained from krill shell by simple extraction with cheap inorganic compounds (HCl and KOH), whereas polyethylene must be polymerized with ethylene (petroleum derivative) and then functionalized by γ-radiation.[11,12] Thereby, a balance must be reached among soluble/immobilized forms and polyethylene/chitin supports.

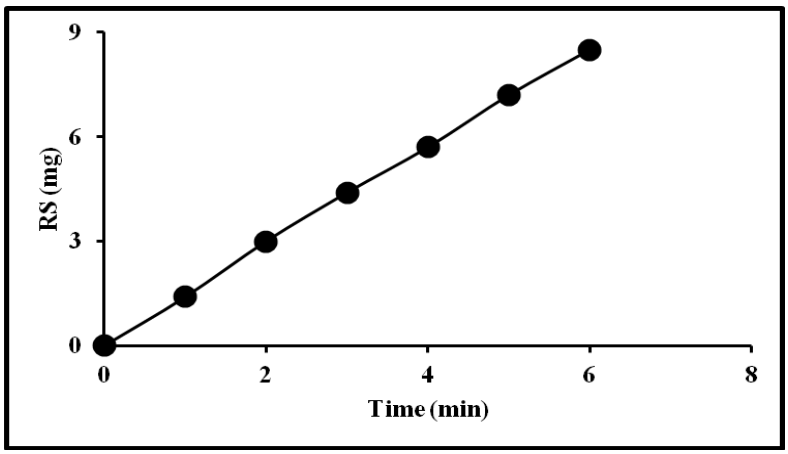

Figure 5: Formation of reducing sugars versus reaction time during sucrose hydrolysis by soluble invertase.

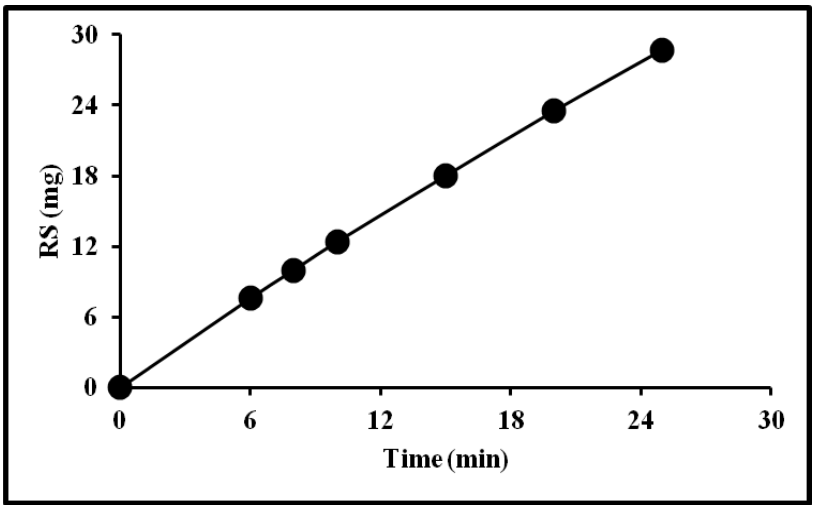

Figure 6: Formation of reducing sugars versus reaction time during sucrose hydrolysis by invertase immobilized on polyethylene.

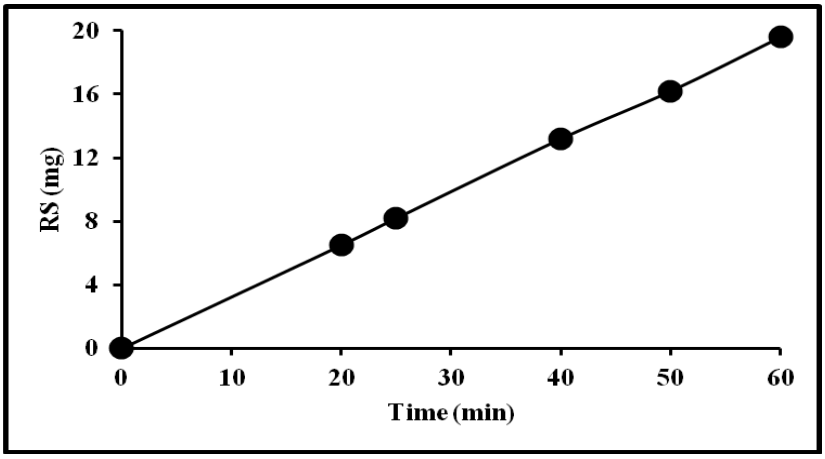

Figure 7: Formation of reducing sugars versus reaction time during sucrose hydrolysis by invertase immobilized on chitin.

The kinetic constants for SI, ICI, and IPI were calculated by using the Hanes-Woolf plot method (Figure 8). The minimum square linear regression equations were:

$(S/v)_{SI} = 14.26 + 0.370.(S)$ $(r = 0.9991)$ (Eq. 11)

$(S/v)_{IPI} = 43.5 + 0.845.(S)$ $(r = 0.9996)$ (Eq. 12)

$(S/v)_{ICI} = 100.3 + 3.17.(S)$ $(r = 0.9992)$ (Eq. 13)

Thereby, the kinetic constants values were: **a) SI** (K_M = 38.5 mM; V_{max} = 2.7 U/mg of protein); **b) IPI** ((K_M = 51.4 mM; V_{max} = 1.2 U/mg of protein); **c) ICI** (K_M = 31.6 mM; V_{max} = 0.32 U/mg of protein).

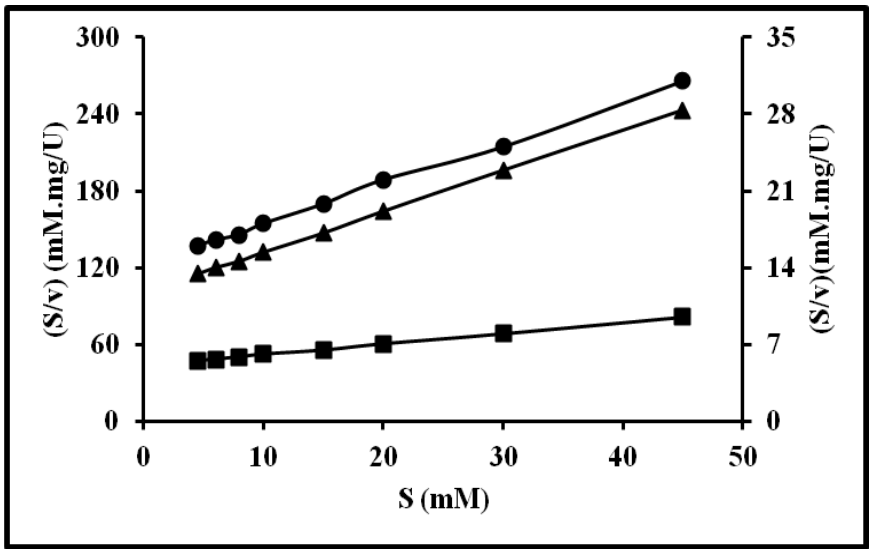

Figure 8: Hanes-Woolf plot for calculating the kinetic constants of soluble invertase (SI; ●), chitin immobilized invertase (ICI; ▲), and polyethylene immobilized invertase (IPI; ■).

The highest activities for SI, ICI, and IPI occurred at pH 5.0, 4.0, and 4.6, respectively (Figure 9). Clearly, the optimum pH for ICI and IPI activities were shifted to the acid pH range when compared to SI. This is the result of H_3O^+ partition between the surroundings of invertase-chitin or polyethylene-invertase complex and the bulk of solution. As the pH values shown in Figure 9 were measured with the glass-pH-electrode immersed in the bulk solution, so the acidic character of this part of the system was due to the increase of H_3O^+ concentration. Chitin and polyethylene supports that had a net positive electrostatic charge repelled the H_3O^+ from the surroundings of the support particles to the bulk. Partition of charged substances between the microenvironment surrounding the immobilized enzyme and the bulk of solution is quite common when charged supports are employed.[7]

Noteworthy, SI and ICI stability occurred, respectively, at pH intervals of 4.6-5.6 and 4.0-6.0, while IPI stability occurred at pH 4.6 (Figure 10). Differently of SI and ICI, the reaction catalyzed by IPI must be rigorously controlled regarding its pH, otherwise the invertase catalytic activity is irreversibly reduced by 33% (pH 4.0) or 20% (pH 5.0) (Figure 10).

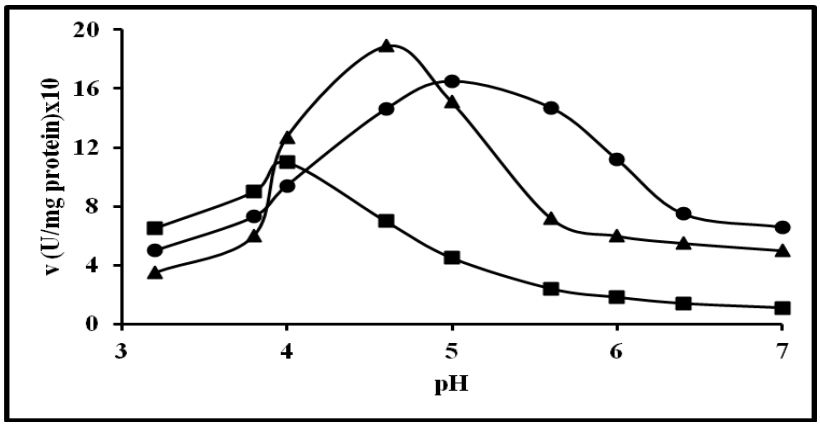

Figure 9: Variation of SI (●), ICI (■), and IPI (▲) activity against pH.

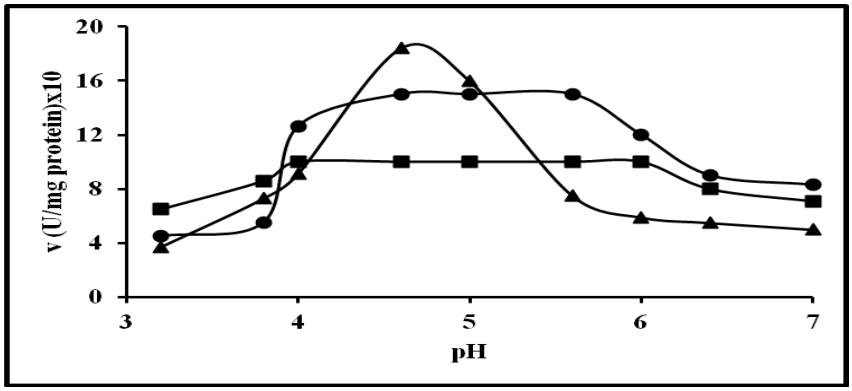

Figure 10: Variation of SI (●), ICI (■), and IPI (▲) stability against pH.

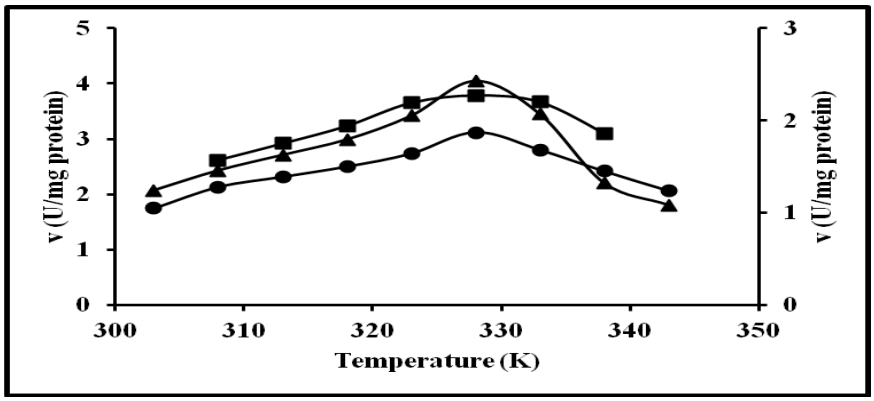

Figure 11: Variation of SI (●), ICI (■), and IPI (▲) activity as a function of temperature. The total reaction times at each temperature for sucrose hydrolysis were 10 min (SI) and 30 min (ICI and IPI).

The activation energy (E_a) for each form of invertase was calculated by using the Arrhenius method, being the minimum square linear regression equations:

$\text{Ln } v_{SI} = 5.78 - 1{,}706.2 \times 1/T \quad (r = -0.991) \quad \text{(Eq. 14)}$

$\text{Ln } v_{ICI} = 5.15 - 1{,}446.1 \times 1/T \quad (r = -0.995) \quad \text{(Eq. 15)}$

$\text{Ln } v_{IPI} = 8.91 - 2{,}479.7 \times 1/T \quad (r = -0.997) \quad \text{(Eq. 16)}$

Where: v_{SI}, v_{ICI}, and v_{IPI} are the activities of invertase forms.

Gibbs free energy (ΔG), enthalpy (ΔH), and entropy (ΔS) were calculated by equations:[13]

$\Delta G = (R.T/2.303).\ \text{Log}\ (v.h/k.T) \quad \text{(Eq. 17)}$

$\Delta H = E_a - R.T \quad \text{(Eq. 18)}$

$\Delta S = (\Delta H - \Delta G) \div T \quad \text{(Eq. 19)}$

Where: h (Plank constant) = 3.978×10^{-32} (J.min); k (Boltzman constant) = 1.38×10^{-23} ($J.K^{-1}$); R (Clapeyron constant) = 8.31 ($J.K^{-1}.mol^{-1}$); T = absolute temperature (K).

The values of thermodynamic parameters are presented in Table 7.

Table 7: Parameters related to SI, ICI, and IPI.

Parameter	SI	ICI	IPI
K_M (mM)	38.5	31.6	51.4
V_{max} (U/mg protein)	2.7	0.32	1.2
pH_{opt}	5.0	4.0	4.6
T_{opt} (K)	328	328	328
E_a (kJ/mol)	14.2	12.0	20.6
ΔG (kJ/mol)	- 67.7	- 67.1	-65.5
ΔH (kJ/mol)	11.5	9.3	17.9
ΔS (kJ/mol.K)	0.24	0.23	0.25

CONCLUSION

The data led us to conclude that fed-batch fermentation was adequate for circumventing the catabolite repression caused by sucrose on yeast invertase formation. The exponentially increasing feeding mode (t_f = 2 h, ΔV = 1 L, t_g = 4 h, 500 rpm, pH 4.5, 35 ^oC, and 30 g of sucrose) led to an invertase productivity of 5.22 U_Y/L. Invertase was extracted from cells obtained under the cited conditions following a downstream protocol that led to a final purification factor of 6.6. Then, invertase was immobilized on chitin (ICI) and polyethylene (IPI), having a hydrolytic activity of 0.326 U_{ICI}/mg protein and 1.14 U_{IPI}/mg protein, respectively. The activity of soluble invertase (SI) was 1.42 U_{SI}/mg protein. The Gibbs free

energy value (-67.7 kJ/mol) for all forms of invertase showed that immobilization did not affect the invertase catalytic performance.

Funding: This work was supported by the National Council for Scientific and Technological Development – CNPq (grant No. 303082/2015-1).

REFERENCES

1. Vitolo M. Invertase activity of intact *Saccharomyces cerevisiae* cells cultured in sugarcane molasses by batch fermentation process. World Journal of Pharmacy and Pharmaceutical Sciences, 2019; 8(3): 126-137.
2. Vitolo M. Circumventing the glucose repression on invertase activity of Baker's yeast by using fed-batch fermentation. World Journal of Pharmacy and Pharmaceutical Sciences, 2019; 8(4): 125-132.
3. Vitolo M. Effect of pH on ethanol and invertase production by *Saccharomyces cerevisiae* grown in blackstrap molasses. World Journal of Pharmacy and Pharmaceutical Sciences, 2019; 8(10): 108-116.
4. Kulshrestha S, Tyazi P, Sindhi V, Yadsville KS. Invertase and its applications – a brief review. Journal of Pharmacy Research, 2013; 7: 792-797.
5. Vitolo M. Enzymes: the catalytic proteins. In: Vitolo M (ed.). Pharmaceutical Biotechnology. São Paulo, Blücher, 2015; 203-228.
6. Vitolo M. Adsorption of invertase on anionic resin. European Journal of Pharmaceutical and Medical Research, 2020; 7(7): 113-117.
7. Vitolo M. Calcium-alginate beads as carriers for biocatalyst encapsulation. World Journal of Pharmaceutical Research, 2019; 8(10): 1-17.
8. Vitolo M. Overview on elemental kinetic of microbial growing. World Journal of Pharmaceutical Research, 2020; 9(15): 50-71.
9. Vitolo M. Membrane reactor applied to enzyme conversion of sucrose and glucose. World Journal of Pharmacy and Pharmaceutical Sciences, 2020; 9(9): 43-70.
10. Rocha-Filho JA, Vitolo M. Guide for practical classes in enzyme and fermentation biotechnology. Edgard Blücher, São Paulo, Brazil, 2017.
11. Barros DP, Vitolo M. Sucrose hydrolysis by invertase immobilized on chitin. Lebensmittel-Wissenchaft und Technologie, 1992; 25: 240-243.

12. De Queiroz AAA, Vitolo M, Oliveira RC, Higa ZO. Invertase immobilization onto radiation-induced graft copolymerized polyethylene pellets. Radiation Physics and Chemistry, 1996; 47(6): 873-880.
13. Owusu RK, Makhzoum A. Heat inactivation of lipase from psychrotrophic *Pseudomonas fluorescens* activation parameters and enzyme stability at low and ultra-high temperatures. Food Chemistry, 1992; 44: 261-268.
14. Zajik EJ, LeDuy A. A geometrical approach for differentiation of an experimental function at a point: applied to growth and product formation. Biotechnology and Bioengineering, 1973; 15: 805-810.
15. Meyenburg HK. The budding cycle of *Saccharomyces cerevisiae*. Pathology Microbiology, 1968; 31: 117-127.
16. Lievense JC, Lim HC. The growth and dynamics of *Saccharomyces cerevisiae*. Annual Report Fermentation Processes, 1982; 5: 211-262.
17. Andrews BJ, Herskowitz I. Regulation of cell cycle-dependent gene expression in yeast. Journal Biological Chemistry, 1990; 265(24): 14057-14060.
18. François J, Eraso P, Gancedo C. Changes in the concentration of cAMP, fructose 2,6-bifosfatase and related metabolites and enzymes in *Saccharomyces cerevisiae* during growth on glucose. European Journal Biochemistry, 1987; 164: 369-373.
19. Smith ME, Dickinson JR, Wheals AE. Intracellular and extracellular levels of cAMP during the cell cycle of *Saccharomyces cerevisiae.* Yeast, 1990; 6: 53-60.