THERMAL STABILITY OF THE IMMOBILIZED FRUCTOSYLTRANSFERASE FROM *Rhodotorula* sp.

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Abstract - The thermal stability of the extracellular fructosyltransferase (FTase) from *Rhodotorula* sp., recovered from cultivation medium by ethanol precipitation and immobilized onto niobium ore, was studied by Arrhenius plot, half-life profile, half-inactivation temperature (T_{50}) and thermodynamic parameters. The Arrhenius plot showed two different behaviors with different deactivation energies (E_{ad}) only after immobilization, the transition occurring in the temperature interval between 51 and 52°C. T_{50} for the free enzyme was estimated to be around 62°C and, after immobilization, 66°C. After 15 minutes at 52°C, it was also possible to observe enzymatic activation for both the free and immobilized forms, but greater activation was achieved at pH 4.5 with the immobilized enzyme. Between 47–51°C the immobilized enzyme was more stable than the free enzyme, with pH 6.0 being the more stable condition for the immobilized enzyme. However, above 52°C the free form was more stable.
Keywords: Niobium; Adsorption; Half-life; Thermal activation; Arrhenius plot; Dimer.

INTRODUCTION

Fructooligosaccharides (FOS) belong to the functional class of foods, due mainly to their prebiotic properties (Gibson *et al.*, 2004). FOS can be naturally obtained from fruits, greens and vegetables (Tanriseven and Aslan, 2005) and also from the enzymatic hydrolysis of inulin and/or the transfructosylation of sucrose residues by certain microbial enzymes; the most well-known and most widely applied enzymes in FOS production are those obtained from filamentous fungi and certain bacteria. Nevertheless**,** yeasts of the genus *Rhodotorula,* isolated from Brazilian Atlantic Forest flowers in the authors' laboratory (Maugeri and Hernalsteens, 2007), are capable of extracellularly producing the dimeric enzyme fructosyltransferase (FTase, EC 2.4.1.9), which presents high transfructosylation potential (Hernalsteens and Maugeri, 2008).

The immobilization of this enzyme on a solid-acid niobium-graphite compound has been the subject of several studies, such as: characterization of the immobilized fructosyltransferase (Aguiar-Oliveira and Maugeri, 2010), studies of the thermostability (reported in the present paper), kinetic studies (Alvarado-Huallanco and Maugeri-Filho, 2010) and the optimization of FOS synthesis in shaking flasks (unpublished data). Initial studies of the characterization of the free enzyme obtained in a synthetic medium (GYMP) showed an optimum pH value of 4.5 (Hernalsteens and Maugeri, 2008). Studies performed with the same enzyme, but obtained using an industrial medium (containing industrial by-products such as corn steep liquor and sugar cane molasses) and immobilized on the same support, showed several effects caused by the strong negative charge of the support on the enzyme, such as the existence of two

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distinct points with maximum activity: pH 4.5 - similar to the free enzyme - and pH 6.0 - presenting excellent thermal stability (Aguiar-Oliveira and Maugeri, 2010).

The extracellular frustosyltranferase (FTase) from *Rhodotorula* sp. is obtained by only one purification step (ethanol precipitation – partially purified enzyme) since the transfructosylation activity and degree of purity present in the final concentrated enzymatic solution were adequate for the proposed purpose, allied with the fact that the methodology is of low cost and easy to reproduce in an industrial environment (Aguiar-Oliveira and Maugeri, 2010; Hernalsteens and Maugeri, 2008). In addition, kinetics studies with the immobilized FTase showed that there is no significant improvement in sucrose conversion between purified and partially purified enzyme (Alvarado-Huallanco and Maugeri-Filho, 2010).

The industrial application of biocatalysts relies strongly on their stock and operational stabilities. The half life, which expresses the stock stability, provides information regarding the biocatalytic maintenance capacity between manufacturing time and usage. On the other hand, the operational stability describes the enzyme persistence during the process under the conditions of use (Papinutti *et al.*, 2008; Ó'Fágáin, 2003). Immobilization also changes the denaturation profile in both negative and positive ways, one explanation for this being that the decrease in the solubility of the enzyme reduces, for instance, molecular and submolecular mobility and vibrations (Bromberg *et al.*, 2008).

The mechanism of enzyme denaturation can have different stages and complexities, the most common models, described by first order kinetics, being those developed by Arrhenius and/or Lumry & Eyring (Fogler, 1999; Lumry and Eyring, 1954). However, in some cases, an apparently simple, first-order inactivation can mask a set of complex molecular events. It is known that the unfolding of the tertiary structure of proteins leads to a loss of enzyme functionality, resulting in its denaturation (Ó'Fágáin, 1997). Enzyme thermostability studies provide information on the capacity of the enzyme to resist thermal unfolding in the absence of substrate and thermo-affinity studies assess the capacity of the enzyme to perform its function in the presence of the substrate (Nadeem *et al.*, 2007). This work presents the results of thermal stability studies of immobilized fructosyltransferase from *Rhodotorula* sp., which has great potential and viability for fructooligosaccharides (FOS) production, providing valuable information necessary for bioreactor design.

MATERIALS AND METHODS

Materials

As reported in a previous work (Aguiar-Oliveira and Maugeri, 2010), the strain of *Rhodotorula* sp. (LEB-V10: Laboratory of Bioprocess Engineering, UNICAMP, Brazil) was isolated from the Brazilian Atlantic Forest (Maugeri and Hernalsteen, 2007). The industrial substrates for the cultivation medium (by-products) were: sugar cane molasses and corn steep liquor, kindly provided by The Usina Estér (Campinas-SP/Brazil) and Corn Products (Mogi Guaçu-SP/Brazil), respectively. The glucose (hydrolytic activity) was determined by using an enzymatic glucose-oxidase kit from LaborLab® (Guarulhos/São Paulo, Brazil). The solid-acid niobium-graphite (~95% Nb) compound, used as the support, was provided by the Brazilian Company of Metallurgy and Mining (CBMM; Brazil). All the other reagents used were acquired from reliable commercial sources.

Rhodotorula sp. Cultivation and Fructosyltransferase Partial Purification

The conditions for cultivation of the *Rhodotorula* sp. and partial purification of the fructosyltransferase (FTase) from the medium (by precipitation with anhydrous ethanol) have been described previously (Aguiar-Oliveira and Maugeri, 2010). The concentrated enzymatic solution with high transfructosylation activity, used in all experiments, corresponds to ethanol-precipitated enzyme re-suspended in 50 mM acetate buffer, pH 4.5.

Inorganic Support and Enzyme Immobilization

Particles of the solid-acid niobium-graphite compound between 65 and 80 mesh (180 – 212 μm) were obtained and cleaned according to methodology described in previous work (Aguiar-Oliveira and Maugeri, 2010) where it is possible to obtain more information about the mechanical and chemical properties of these particles.

The standard methodology for immobilization by adsorption onto the niobium particles applied in this work was described earlier (Aguiar-Oliveira and Maugeri, 2010; Maugeri and Aguiar-Oliveira, 2007).

The Enzymatic Activity and Related Parameters

As applied and described earlier (Aguiar-Oliveira and Maugeri, 2010; Hernalsteens and Maugeri,

2008), the enzymatic activity of fructosyltransferase (FTase) was determined based on the methods of Hidaka *et al.* (1998) and Chen and Liu (1996). One transfructosylation activity unit (U_{TF}; free enzyme; Eq. (1)) was defined as 1 µmol of fructose transferred (F_T) in one minute (min) and one immobilized activity unit (U_i) expressed as one transfructosylation activity unit immobilized on one gram (g) of support (Eq. (2)).

$$U_{TF} = F_T \ [\mu mol/min] \qquad (1)$$

$$U_i = U_{TF}/g \ [\mu mol/min.g] \qquad (2)$$

The efficiency of immobilization (ε_{immob}) was expressed as the ratio between the enzymatic activity effectively immobilized (U_i) and the total enzymatic activity submitted to immobilization (U_o), as shown in Eq. (3); by definition, U_o corresponds to the ratio between the enzymatic activity of the concentrated solution (U_{TF}) submitted to immobilization and the weight of the support (g). The residual activity at each time (R_T^t) was determined according to Eq. (4) to monitor enzymatic activity losses with time for both the immobilized and free enzymes. For this purpose, some definitions were employed: U_i^o and U_{TF}^o correspond to the initial enzymatic activities determined at time zero (t_o), and U_i^t and U_{TF}^t correspond to the enzymatic activities determined at time t.

$$\varepsilon_{immob} = \frac{U_i}{U_o} \qquad (3)$$

$$R_{T(^oC)}^{t(min)} = \frac{U_i^t}{U_i^o} = \frac{U_{TF}^t}{U_{TF}^o} \qquad (4)$$

Effects of Temperature

The thermostabilities of the free and immobilized fructosyltransferase were followed and established from their residual activities ($R_{T(^oC)}^{t(min)}$) in stocks maintained in 50 mM acetate buffer at pH values of 4.5 and 6.0 [in accordance with Hernalsteens and Maugeri (2008) for the free form and Aguiar-Oliveira and Maugeri (2010) for the immobilized form] in the absence of substrate, for various periods of time at temperatures ranging from 47 to 70°C. All the experiments were performed in triplicate.

Half-inactivation temperatures (T_{50}) and the thermal activation of the free and immobilized enzyme were determined by submitting them to a brief incubation period (in 50 mM acetate buffer without substrate) at

different temperatures (ranging from 47 to 70°C) for 15 min, followed by immersion in an ice bath for 5 min; enzymatic activities were then determined according to 2.4. The enzymatic activity obtained by this procedure corresponds to U_i^{15min} for the immobilized enzyme, and U_{TF}^{15min} for the free enzyme. As a control, the enzymatic activities of free and immobilized enzyme with no incubation were determined and correspond to the values of U_{TF}^o and U_i^o, respectively. These enzymatic activities were used in Eq. (4) to obtain the residual activity as a function of the incubation temperature [(R_T^{15min}) versus T (°C)]. For the immobilized enzyme, the assays were carried out at pH 4.5 and 6.0 (Aguiar-Oliveira and Maugeri, 2010), while for the free enzyme, the assays were performed only at the optimum pH value of 4.5. All experiments were performed in triplicate.

General Enzyme Thermal Stability Parameters

a) k_d

The enzyme denaturation constant (k_d; 1/min) was obtained from a graph of ln(R_T^t) versus time (min) (data not presented), corresponding to the value of the slope of the regression line obtained. For this calculation, only regressions obtained with a minimum of 5 points and a value for R^2 above 0.93 were accepted.

b) E_{ad} and $t_{1/2}$

The denaturation activation energy (E_{ad}; kJ/mol) and half-life ($t_{1/2}$; min) of the immobilized enzyme were obtained from the k_d values (Catana *et al.*, 2007; Cornish-Bowden, 1995). According to the Arrhenius equation (Eq. (5)), the k_d values were plotted as [-ln(k_d)] versus [$10^3/T$; (1/K)]. The value referred to as E_{ad} is obtained by multiplying the slope of the regression by the ideal gas constant ($R = 8.314 \times 10^{-3}$ kJ/mol·K).

$$k_d = A_d \left[e^{-E_{ad}/RT} \right] \Rightarrow -\ln(k_d) = \left(\frac{E_{ad}}{R} \right) \frac{1}{T} - \ln(A_d) \qquad (5)$$

Eq. (6), allows the calculation of $t_{1/2}$ (min) from the k_d values and combination of this equation with Equation (5) allows one to express $t_{1/2}$ according to Eq. (7).

$$t_{1/2} = \frac{\ln(0.5)}{-k_d} = \frac{0.6931}{k_d} \qquad (6)$$

$$t_{1/2} = \frac{0.6931}{A_d} e^{(E_{ad}/RT)} \qquad (7)$$

c) T_{50} and Thermal Activation

Based on Eq. (4), the residual free and immobilized activities for each temperature were used to construct the graph of $R_{T(^{o}C)}^{15\min}$ (residual activity after 15 min) versus T (°C). The value of the half-inactivation temperature (T_{50}) was determined with the mathematical software TableCurve^{TM} 2D (SPSS, INC., 1996) by means of an adjustment of the points to a mathematical equation that allows the calculation of the temperature required for $R_{T(^{o}C)}^{15\min} = 0.5$. The values obtained for $R_{T(^{o}C)}^{15\min}$ also provided information on the thermal activation of the enzyme at specific temperatures.

d) The Effect of Pre-Incubation at 52 and 60°C

The temperatures of 52 and 60°C, corresponding to the points of maximum activation observed in the study of T_{50}, were evaluated separately with respect to the intensity of the effect of this activation on enzyme stability. The immobilized enzyme (at pH values of 4.5 and 6.0) and the free enzyme (at pH 4.5), obtained under standard conditions and with initial activities of U_i^o and U_{TF}^o, were first submitted to a short incubation at 52 or 60°C for 15 min and then immersed in an ice bath for 2 min before incubating at 65°C (high temperature) for 35 min, presenting final activities of $U_i^{15-35\min}$ and $U_{TF}^{15-35\min}$. The residual activities obtained in each case, $R_{52-65^{o}C}^{*15-35\min}$ and $R_{60-65^{o}C}^{*15-35\min}$ were compared with the residual activity obtained after incubation at only 65°C for 35 min, $R_{65^{o}C}^{35\min}$. The experiments were carried out in triplicate.

Thermodynamic Parameters of Enzyme Denaturation

The values of k_d and E_{ad}, obtained according to sub-items (a) and (b), were used in the calculations of: Gibbs Free Energy (ΔG; kJ/mol; Eq. (8)), Enthalpy (ΔH; kJ/mol; Eq. (9)) and Entropy (ΔS; J/mol.K; Eq. (10)). For these calculations, the value

employed for Boltzmann constant (k_B) was 1.38×10^{-23} J/K and for the Planck constant (h) 11.04×10^{-36} J.min (Doran, 2002; Blanch and Clark, 1997).

$$\Delta G = -RT \ln\left(\frac{k_d.h}{k_B.T}\right) \qquad (8)$$

$$\Delta H = E_{ad} - RT \qquad (9)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \qquad (10)$$

RESULTS AND DISCUSSION

Calculations of E_{ad} (Arrhenius Plot)

a) Free Enzyme

According to Figure 1 the linear regression of the points in the temperature range assessed (47-70°C) was a good fit ($R^2 = 0.992$), the value for the denaturation activation energy (E_{ad}) being 294.35 kJ/mol. The R^2 values for the residual activity (Eq. (4)) ranged from 0.93 to 0.97.
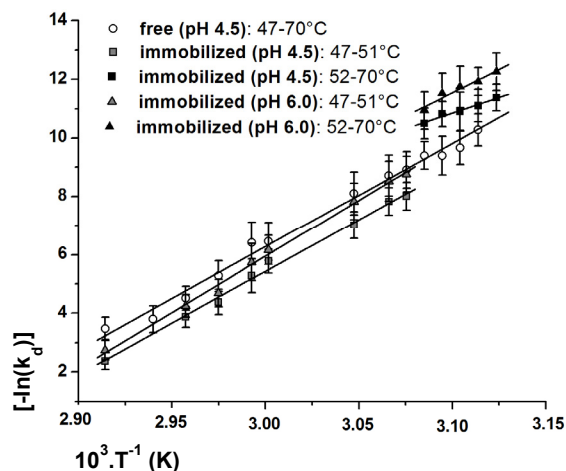

Figure 1: Calculation of E_{ad} for the partially purified fructosyltransferase from *Rhodotorula* sp. in the forms: a) free (pH 4.5) – white circles [linear fit: y = 35.404.x − 99.936; R^2=0.992]; b) immobilized (pH 4.5) phase 1 – black squares [linear fit: y = 35.331.x − 100.56; R^2=0.995] and phase 2 – gray squares [linear fit: y = 21.181.x − 54.807; R^2=0.976]; c) immobilized (pH 6.0) phase 1 – black triangles [linear fit: y = 38.422.x − 86.582; R^2=0.996] and phase 2 – gray triangles [linear fit: y = 31.653.x − 86.582; R^2=0.952].

b) Immobilized Enzyme

Analyzing Figures 1, two distinct phases of the slope can be clearly noted at the two pH values evaluated. The differentiation between the phases occurred in an interval of just 1°C, between 51 and 52°C. The first phase corresponded to the lower temperature range, from 47 to 51°C, and the second phase comprised the temperatures from 52 to 70°C. In the first phase, the linear fit gave E_{ad} values of 175.10 kJ/mol ($R^2 = 0.976$) at pH 4.5 and 263.16 kJ/mol ($R^2 = 0.952$) at pH 6.0; in the second phase (E_{ad}) was calculated as 293.74 kJ/mol ($R^2 = 0.995$) at pH 4.5 and 319.44 kJ/mol ($R^2 = 0.996$) at pH 6.0 The R^2 values for the residual activity (Eq. (4)) ranged from 0.93 to 0.96.

When compared to the free enzyme, the immobilized enzyme presented unusual behavior, since a single slope profile is more commonly observed, as in the case of the free enzyme (Bhatti *et al.*, 2007; Santos *et al.*, 2007). However, unusual profiles have previously been reported for Arrhenius plots, and even a "zig-zag" like profile with two negative slopes and one positive slope has been reported (Šikšnis *et al.*, 1990).

The denaturation activation energy (E_{ad}) values for enzymes generally vary from 170 to 400 kJ/mol (Doran, 2002) and this parameter expresses how much energy is needed to promote enzyme denaturation under the conditions assessed. The E_{ad} values obtained shows that, at pH 4.5, the free and the immobilized fructosyltransferase (in temperature phase 2) presented very similar energy values. With respect to the immobilized enzyme, the value of E_{ad} was higher in temperature phase 2 (52-70°C) for both the pH values assessed. Several authors have shown that chemical modifications of the surface can drastically change enzyme behavior with respect to its thermostability and other aspects of interest, due to conformational changes (Bromberg *et al.*, 2008; Bhatti *et al.*, 2007; Šikšnis *et al.*, 1990). It must be emphasized that immobilization, especially on a strongly loaded support such as niobium, can alter the enzyme behavior or highlight phenomena imperceptible when the enzyme is dispersed in the medium (Sadana, 1998).

The profiles presented here can also be analyzed considering the dimeric form presented by this enzyme (Hernalsteens and Maugeri, 2008) which, according to some authors, is the most common form presented by all fructosyltransferases (Lüscher *et al.*, 1996). It is also possible to find references to the action of two distinct fructosyltransferases (Nemukula *et al.*, 2009). Between 51 and 52ºC, some type of energy differentiation occurs with the immobilized enzyme and, to understand this phenomenon, one must consider the influence of the support and, principally, the form in which the enzyme is immobilized on the support. Some authors defend the idea that, in the case of dimeric enzymes, for example, immobilization can occur with just one of the sub-units, leaving the other one free, and this could alter many aspects of enzymatic functionality (Trevan, 1980). In a study carried out with a trimeric enzyme (Bolivar *et al.*, 2009), a strong argument in favor of the partial immobilization of sub-units was that the dissociation of these sub-units could, in many cases, be the first step in the inactivation of polymeric enzymes, or the desorption of one of the non-immobilized sub-units could occur, contaminating the reactive medium. One can even conjecture that, when incubated at higher temperatures, the molecule of fructosyltransferase from *Rhodotorula* sp. immobilized on niobium could be in the form of two monomers and, in this case, would probably require more energy to denature these two independent units. On the other hand, at lower temperatures, the dimeric form would confer greater stability, requiring a lower value for E_{ad}.

Calculation of $t_{1/2}$

Mathematical expressions for the half life ($t_{1/2}$) at different temperatures, obtained according to Eq. (7), are listed below as Eqs. (11) to (15). With the help of the software Statistica 5.0 (STATSOFT, INC., 2000), the equations were fitted to the experimental points in order to obtain the respective R^2 values.

a) free FTase (pH 4.5) / 47-70°C – $R^2 = 0.992$

$$t_{1/2} = 6.935 \times 10^{-3} e^{35.404/T} \tag{11}$$

b) immobilized FTase (pH 4.5) 47-51 °C – $R^2 = 0.976$

$$t_{1/2} = 1.265 \times 10^{-2} e^{21.181/T} \tag{12}$$

c) immobilized FTase (pH 4.5) 52-70 °C – $R^2 = 0.995$

$$t_{1/2} = 6.892 \times 10^{-3} e^{35.331/T} \tag{13}$$

d) immobilized FTase (pH 6.0) 47-51°C – $R^2 = 0.953$

$$t_{1/2} = 8.005 \times 10^{-3} e^{31.653/T} \tag{14}$$

e) immobilized FTase (pH 6.0) 52-70 °C – $R^2 = 0.995$:

$$t_{1/2} = 6.340 \times 10^{-3} e^{38.422/T} \tag{15}$$

As discussed before, for the free enzyme the profile is exponential and uninterrupted and obeys only Eq. (11). However, for the immobilized enzyme at both pH values (4.5 and 6.0), the differentiation between the profiles is clear in both temperature phases (Figure 2). At 50°C, corresponding to the optimum temperature for FOS synthesis with the free enzyme (Hernalsteens and Maugeri, 2008), the half life ($t_{1/2}$) calculated for the free enzyme was 5.81 days. After immobilization, this value increased to approximately 24 days at pH 4.5 and nearly twice this value at pH 6.0. Therefore, immobilization was beneficial to the enzyme between 47 and 51°C, increasing its stability (Figure 2). This characteristic makes the use of immobilized fructosyltransferase very promising, because if Equations (13) and (15) were followed at temperatures below 51°C, the values estimated for the half life of the immobilized enzyme at pH 4.5 and 6.0 would be greater than 3 and 7 days, respectively. At 48°C and pH 6.0, corresponding to the optimized temperature and pH for FOS synthesis with the immobilized fructosyltransferase (data not published yet), the half life obtained experimentally is 72 days, while that estimated according to Equation (14) is 77 days: for the free enzyme at 48°C, this value is 14 days – from experimental data and according to Eq. (11).

Thermodynamic Denaturation Parameters

Protein unfolding is followed by the rupture of several links, generating a disorganized system. This increase in entropy (ΔS; J/mol.K) is compensated by a decrease in Gibbs free energy (ΔG; kJ/mol), making it easier for denaturation to occur, meaning that, when the susceptibility to denaturation is higher, the enzyme is in a less energetic state and in greater disorder, since its original structure has been destroyed.

The results for the Gibbs free energy (ΔG; kJ/mol) indicate that, at the temperatures of the first phase (47-51°C), the immobilized enzyme has (Figure 3) a more stable intrinsic conformational form, since the values are higher, also indicating that this range is less favorable for protein unfolding. At temperatures above 52°C, the Gibbs energy reached completely different levels (Figure 3). This difference between the temperature ranges can also be seen from the values of the enthalpy (ΔH; kJ/mol) and entropy (ΔS; J/mol.K), which indicate that the susceptibility of the enzyme to thermal denaturation increases with an increase in temperature (Bromberg et al., 2008).

According to some authors, one can apply the concept of $\Delta Cp = 0$ to the thermodynamic analyses of enzyme denaturation processes, leading to the conclusion that ΔH and ΔS are independent of the temperature, as observed in Figure 3 (Doran, 2002; Blanch and Clark, 1997). However, for other authors, this supposition represents a simplistic vision of the phenomenon and they defend a marked temperature-dependence (Becktel and Schellman, 1987). The present authors believe that the results and discussion presented in this paper have attended the proposed objective, which was to provide basic knowledge on the heat denaturation phenomenon of immobilized FTase.
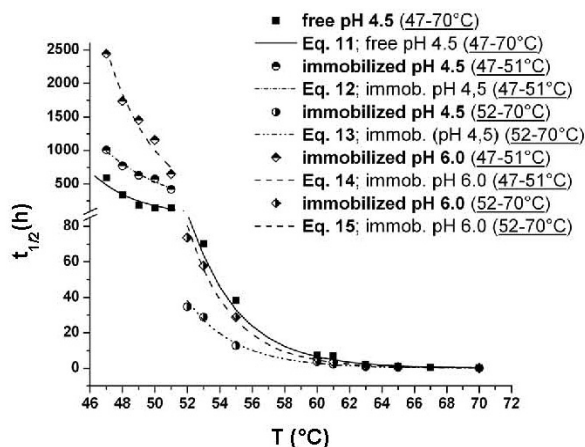

Figure 2: Experimental data (symbols) and estimates according to the model (lines) for the half-life ($t_{1/2}$) of the partially purified fructosyltransferase from *Rhodotorula* sp. in the free form at pH 4.5 (represented by squares and continuous lines) and immobilized at: pH 4.5 (represented by circles and dotted lines) and pH 6.0 (represented by diamonds and dashed lines).
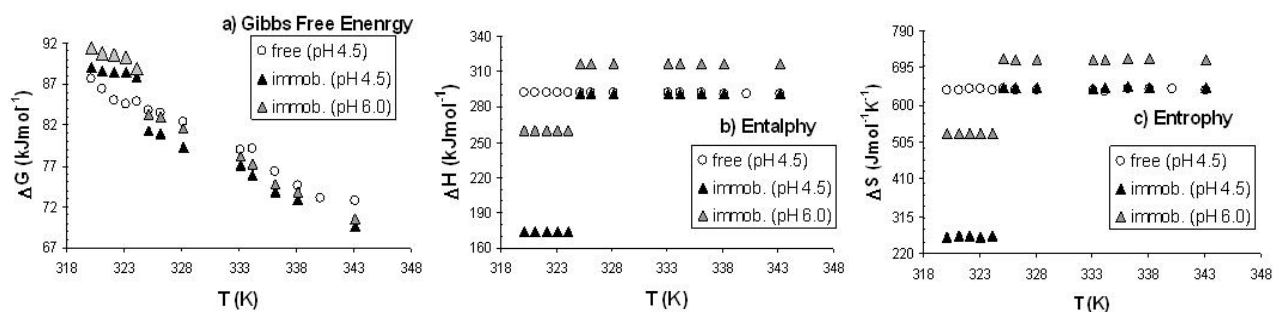
Figure 3: Thermodynamic parameters for the denaturation of the partially purified fructosyltransferase from *Rhodotorula* sp. free (black circles) and immobilized at pH 4.5 (dark grey triangles) and at pH 6.0 (light grey triangles): a) Gibbs free energy (ΔG); b) Enthalpy (ΔH); c) Entropy (ΔS).

T_{50} and Thermal Activation

The mean transition point for thermal protein unfolding can be described as the half-inactivation temperature or unfolding temperature (T_{50}). This parameter corresponds to the temperature at which the residual activity (Eq. (4)) drops to 50% of its initial value after a brief period of incubation, such as 10 (Ruth *et al.*, 2007) or 15 min (Ó'Fágáin, 1997). The basic profile for this type of analysis would be of a sigmoidal type, where, at a certain temperature, the activity decreases significantly, indicating that a brief incubation is sufficient to affect thed thermal stability of the enzyme. This analysis also makes it possible to confirm the effects of thermal activation (Papinutti *et al.*, 2008). The values obtained for $R_{T(^{\circ}C)}^{15\,min}$ are shown in Figure 4.

According to the results, one can identify a moderate activation range for the free enzyme between 51 and 57°C, not exceeding 8% of the initial activity. When analyzing the immobilized enzyme, one can distinguish two temperature ranges at both pH values where enzyme activation was significant, these being 57-61°C and 51-53°C. Comparing pH 4.5 with pH 6.0 (this latter showing greater stability than the former), the effect of activation on the immobilized enzyme was always higher at pH 4.5, which is equivalent to the optimum pH of the free enzyme. The maximum activation point was observed at a temperature of 52°C, where the value for $R_{52^{\circ}C}^{15\,min}$ reached $1.82 \pm 2.2\text{E}^{-2}$ at pH 4.5 and $1.56 \pm 2.1\text{E}^{-2}$ at pH 6.0. As previously discussed in item 3.1, the temperature range between 51 and 52°C suggests some type of energetic and/or conformational limit for the immobilized enzyme, indicating a strong effect of the support.
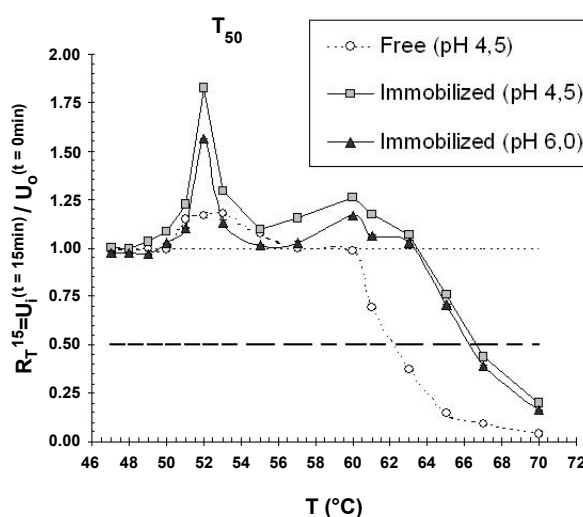

Figure 4: Thermal activation profile after 15 min of incubation at different temperatures and the estimation of the half-inactivation temperature (T_{50}) for the partially purified fructosyltransferase from *Rhodotorula* sp. in the free form (white circles and dotted line) and immobilized form at pH 4.5 (grey squares and full line) and at pH 6.0 (black triangles and full line). The light dotted line parallel ($R_T^{15}=1.0$) is a reference for the standard condition, without previous incubation; the parallel darker dashed line ($R_T^{15}=0.50$) indicates T_{50}.

When the free enzyme was at pH 4.5, there was a pronounced activity loss as of 61°C, the T_{50} of the free enzyme being calculated as 62.06°C. For the immobilized enzyme, the negative effect of incubation became evident at temperatures above 65°C, the T_{50} being estimated as 66.57ºC at pH 4.5 and 66.21ºC at pH 6.0. However, according to the discussion presented in item 3.1, immobilization was observed to affect enzyme thermostability, at least in

the temperature range from 52-70°C. Nevertheless, Figure 4 shows that, at temperatures above 63-65°C, the effect of a brief incubation is greater for the free enzyme. Possible explanations for this could be the existence of some type of mild activation caused by the support, even at high temperatures, or maybe some type of protection caused by immobilization.

For some enzymes, a pre-incubation step increases stability and is frequently recommended (Papinutti *et al.*, 2008). The present study of the influence of pre-incubating the free and immobilized fructosyltranferase at the temperatures of greatest thermal activation showed that neither of the temperatures evaluated (52 and 60ºC) increased thermal stability, free or immobilized, at a higher temperature (65ºC), as can be seen from the values for the residual activity in Table 1. With pre-incubation at both temperatures, the free residual activity was about 70% lower than without pre-incubation. For the immobilized enzyme, pre-incubation at 52ºC gave a reduction of 17% as compared to the residual value without incubation, and of 35% at 60ºC. At pH 6.0, the immobilized enzyme showed the worst effects, with a reduction of approximately 65% at both temperatures evaluated.

Table 1: The effect of pre-incubation at 52 or 60 ºC on the thermal stability of free (pH 4.5) and immobilized (pH 4.5 and 6.0) partially purified fructosyltransferase from *Rhodotorula* sp. expressed as a function of the residual activity (Eq. (4)) with (R^{*15-35}_{T-65}) and without (R^{35}_{65}) the pre-incubation step.

	Free (pH 4.5)	Immobilized (pH 4.5)	Immobilized (pH 6.0)
R^{35}_{65}	0.680 ± 0.003	0.485 ± 0.002	0.606 ± 0.003
R^{*15-35}_{52-65}	0.209 ± 0.003	0.400 ± 0.018	0.210 ± 0.007
R^{*15-35}_{60-65}	0.200 ± 0.001	0.316 ± 0.020	0.217 ± 0.002

CONCLUSIONS

With respect to the thermostability of the extracellular fructosyltransferase (FTase) from *Rhodotorula* sp. (LEB-V10: Laboratory of Bioprocess Engineering, UNICAMP, Brazil), it can be concluded that the immobilization by adsorption on niobium ore (a solid-acid compound) significantly improved the enzymatic stability, especially at temperatures lower than 51°C and at pH 6.0, a value less acidic than the optimum condition for the free enzyme (pH 4.5). The results presented so far indicate that the support (niobium) and the immobilization technique (adsorption) can affect in

different ways each one of the subunits of immobilized fructosyltransferase, or both at the same time. This is indicated by two distinct optimum pH conditions, by the effect of the specific temperature of 52°C on thermal stability as observed by the energetic differentiation between 51 and 52°C, and additionally by the enzymatic activation observed after a pre-incubation, especially at pH 4.5 for the immobilized enzyme. How and what parts of the subunit(s) are involved in the adsorption of the molecule are important knowledge needed for a full understanding of the enzymatic behavior that is observed only after immobilization. The most plausible explanation for these differences between the free and immobilized enzymes, presented in this paper, is the effect of the support on the enzymatic micro-environment. We believe that it is possible that the subunits dissociate and remain immobilized and it is also possible that the molecular conformation modifies in order to adapt to the changes in the macro-environment, like pH or temperature changes. Several authors like Dosztányi *et al.* (1997) have studied how to predict or locate the specific sites on protein structures that define their behavior and stability. Therefore, subsequent studies of the molecular conformation of free and immobilized fructosyltransferase are crucial in order to complete and explain the results presented in this paper, which are the first results for the thermal stability of the extracellular fructosyltransferase from *Rhodotorula* sp. immobilized on niobium and represent (together with our first publication: Aguiar-Oliveira and Maugeri, 2010) the beginning of a sequence of studies related to this enzyme and its biotechnological application.

NOMENCLATURE

E_{ad}	Denaturation activation energy	kJ/mol
F_T	Fructose transferred	μmol
FTase	Fructosyltransferase	
FOS	Fructooligosaccharides	

k_B	Boltzmann constant	J/K
$\underline{k_d}$	Denaturation constant	1/min
H	Planck constant	J.min
R	Ideal gas constant	$kJ/mol^{.}K$
$t_{1/2}$	Half-life	min, days
T_{50}	Half-inactivation temperature	°C
U_o	Enzymatic activity submitted to immobilization	μmol/min
U_i	Immobilized activity unit	μmol/min.g
U_{TF}	Transfructosylation activity unit	μmol/min
R_T^t	Residual activity at temperature "T (°C)" and time "t (min)"	
U_i^o, U_{TF}^o	Initial enzymatic activities at time zero t_o	
U_i^t, U_{TF}^t	Initial enzymatic activities at time t (min)	
ΔG	Gibbs free energy	kJ/mol
ΔH	Enthalpy	kJ/mol
ΔS	Entropy	J/mol.K
ε_{immob}	Efficiency of immobilization	

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