ISSN 1330-9862
(FTB-2318)

original scientific paper

Effect of Organic Solvent on the Characteristics of Free and Immobilized Inulinase from *Kluyveromyces marxianus* ATCC 16045

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Received: July 7, 2009
Accepted: February 1, 2010

Summary

The aim of this work is to evaluate the effects of the butyl acetate concentration on the characteristics of free and immobilized inulinase from *Kluyveromyces marxianus* ATCC 16045. The mass fractions of organic solvent (OS) in sodium acetate buffer (0.1 M) were studied in the range from 25 to 70 %. The characteristics of both free and immobilized enzymes were not significantly affected by the OS mass fraction. The optimal temperature for the free enzyme was 55 °C at all OS mass fractions studied, whereas for the immobilized enzyme the optimum was 55 °C at 70 % of butyl acetate, and in the range from 50 to 60 °C at 25 and 50 % of OS. The optimum pH values, at all OS mass fractions, were 4.8 and 4.4 for the free and immobilized enzymes, respectively. The immobilized enzyme showed more stability at 50 °C and pH=4.8 for the whole range of OS mass fractions, since its stability was improved about 3 times. The kinetics parameters were calculated using Lineweaver-Burk plots. For the free enzyme, the v_{max} values were 12.5, 58.5 and 37.6 U/mL and the K_m values 17.5, 280.7 and 210.4 mM at butyl acetate mass fractions of 25, 50 and 70 %, respectively. Similarly, for the immobilized enzyme, the v_{max} values were 38.9, 59.5 and 72.5 U/mL and the K_m values 3.1, 5.4 and 14.0 mM at the same butyl acetate mass fractions, respectively.

Key words: organic solvent, inulinase, stability, kinetic parameters

Introduction

Microorganisms are essential in the food industry because they are sources of many additives and processing aids used in food processing. Currently, more than 50 different enzyme products are being employed in food processing (*1*,*2*). Amongst the used enzymes, inulinase plays an important role in the production of high fructose syrups (HFS) by means of enzymatic hydrolysis of inulin (*3*), and in the synthesis of fructooligosaccharides, since the enzyme has significant transfructosylating activity at high sucrose concentrations (*4*).

Although the enzymes show excellent catalytic properties, these properties usually have to be improved before their implementation on an industrial scale, where many cycles of high yield processes are desired. Generally, soluble enzymes are immobilized so that they can be reused many times and, in addition, some other critical enzyme properties have to be improved, such as stability, activity, inhibition by reaction products and selectivity towards non-natural substrates (*5*).

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In addition, interactions between an enzyme molecule and the surrounding liquid are of crucial significance for enzymatic catalysis. Some authors have stated that enzymes in anhydrous non-aqueous solvents exhibit remarkable novel properties, such as greatly enhanced stability, radically altered substrate specificity, and the ability to catalyze new reactions (*6*,*7*). However, at high organic solvent mass fractions, the enzyme can be deactivated, because of reversible conformational changes in the protein molecule. An alternative way to overcome this problem is the use of immobilized enzymes, since the immobilization prevents protein-protein interactions that lead to aggregation of the protein molecules and hence to irreversible deactivation (*5*).

The success of the design of an enzymatic reactor depends on the enzyme behaviour during the process, the technical and economical viability of the enzyme immobilization and the enzyme stability. The modifications of the optimal temperature and pH, and the effects of temperature and pH on the enzyme stability can occur as a consequence of immobilization and/or the use of an organic solvent. Some studies provide useful information about the relationship between the enzyme protein structure and the enzyme activity, and about the physical and/or chemical properties of the carrier surface (*8*).

In this context, the aim of the present work is to study the activity, stability, Michaelis constant (K_m) and the maximum rate of sucrose hydrolysis reaction (v_{max}) of free and immobilized inulinase in aqueous butyl acetate solutions, and to investigate the effects of the organic solvent mass fraction on these parameters. The inulinase used in this study was obtained from the strain *Kluyveromyces marxianus* ATCC 16045.

Material and Methods

Microorganism, medium and fermentations

Kluyveromyces marxianus ATCC 16045 was employed to produce the inulinase since it fulfils the requirements for GRAS (generally recognized as safe) and is accepted by the FDA (Food and Drug Administration). The microorganism was grown on malt-yeast extract (MY) agar medium containing (in g/L): yeast extract (Difco Laboratories, USA) 3.0, malt extract (Difco Laboratories, USA) 3.0, peptone (Difco Laboratories, USA) 5.0, glucose (Synth, Brazil) 10.0 and agar (Merck, Germany) 20.0, and was subcultured every three weeks. To produce cells for the pre-inoculum, the culture was grown for 24 h in 500-mL flasks with 100 mL of MY medium without agar, containing 20 g/L of sucrose at pH=6.8, 30 °C and 150 rpm.

Fermentations were carried out in 500-mL conical flasks with 100 mL of culture medium containing (in g/L): sucrose 14.0, yeast extract 10.0, peptone 20.0 and K_2HPO_4 1.0. Inoculum of 10 % (by volume) was used with an initial pH of 3.5, 30 °C and agitation at 150 rpm in an orbital shaker (Psycrotherm, New Brunswick Scientific, USA) for 72 h (*9*).

Enzyme recovery

Crude fermented broth was centrifuged for 10 min at 5000×*g* and 5 °C. To precipitate the inulinase, a 70 % ethanol solution (by volume) at 4 °C was pumped into the supernatant at a flow rate of 0.8 mL/min. The supernatant was discarded and the precipitate re-suspended in sodium phosphate buffer (0.05 M and pH=6.0).

Inulinase immobilization

The inulinase was immobilized according to the following methodology: initially a gel solution was prepared containing 16.5 g of distilled water and 0.75 g of sodium alginate, and maintained under mild heating. After complete dissolution of the alginate, 12.5 g of sucrose were added, followed by 5 mL of the solution containing the recovered inulinase, 3.5 mL of glutaraldehyde and 0.75 g of activated carbon.

For sphere formation, the gel solution was pumped into a 0.2 M calcium chloride solution in sodium acetate buffer (0.1 M and pH=4.8) containing 3.5 % of glutaraldehyde, and stirred slowly at 10 °C. The immobilized inulinase was maintained at 4 °C for 24 h and then washed with sodium acetate buffer (0.1 M and pH=4.8). To maintain the structure, the immobilized spheres (around 0.5 cm in diameter) were immersed in a 0.2 M calcium chloride solution in sodium acetate buffer (0.1 M and pH=4.8).

Effect of organic solvent on the characteristics of free and immobilized inulinase

The characteristics of free and immobilized inulinase in the organic solvent were evaluated in terms of the temperature and pH, Michaelis constant (K_m) and the maximum rate of sucrose hydrolysis reaction (v_{max}). The influence of 25, 50 and 70 % (by volume) butyl acetate on the above mentioned parameters was investigated. Butyl acetate was chosen based on preliminary studies of our research group (data not shown).

Optimal temperature and pH

For the determination of optimal temperature, free and immobilized inulinase were incubated in sodium acetate buffer (0.1 M and pH=4.8) for three organic solvent (OS) mass fractions, at temperatures ranging from 30 to 70 °C. To determine the optimal pH, the temperature was maintained at 50 °C and the pH varied from 4.0 to 5.6 in sodium acetate buffer (0.1 M). Enzymatic activity was determined in both cases.

Effect of temperature and pH on enzyme stability

The isothermal inactivation treatment was carried out in a water bath, according to the procedure defined in the preceding paragraph. In the present study, it was assumed that the enzyme inactivation was influenced by pH and temperature, following the first order kinetics, as previously stated by several authors (*10*,*11*). Under isobaric-isothermal conditions, the decrease in enzyme activity as a function of treatment time can be described by the following equation:

$$A = A_0 \exp(-kt) \qquad /1/$$

where k is the inactivation rate constant, and A_0 and A are the initial and residual enzyme activities, respectively, after heating for time t. After determining the k value by non-linear regression, it is possible to calculate

the enzyme half-life. Based on the Arrhenius plots, it is possible to relate temperature to the deactivation energies.

Kinetic studies

Enzyme assays using 1.0 mL of enzyme extract were carried out in sodium acetate buffer (0.1 M and pH=4.8) at 50 °C, with increasing concentrations of sucrose from 0.5 to 35 g/L. The K_m and v_{max} were determined by Lineweaver-Burk plots of $1/v$ *versus* $1/S$, which yields a straight line with an intercept on the y-axis at $1/v_{max}$ and a slope of K_m/v_{max}.

Inulinase assay

Inulinase activity was measured by incubating 1.0 mL of the diluted enzyme source or 1 mL of particles for the immobilized enzyme with 9.0 mL of 2 % (by mass) sucrose solution at 50 °C in sodium acetate buffer (0.1 M and pH=4.8). In the latter case, the volume of particles was measured considering the equivalent volume of water displaced into a graduated tube (*12*). The released reducing sugars were measured by the 3,5-dinitrosalicylic acid method (*13*), and one unit of inulinase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars as glucose per minute under the standard assay conditions.

Results and Discussion

Optimal temperature and pH

Fig. 1 presents the data referring to the inulinase activity as a function of pH and temperature for free and immobilized inulinase at three butyl acetate mass fractions. Optimal temperature for free inulinase was 55 °C (Fig. 1a) and optimal pH=4.8 (Fig. 1b), independent of the organic solvent (OS) mass fraction. For the immobilized inulinase the optimal temperature was 55 °C at 70 % (by mass) of OS (Fig. 1c). The optimal pH was 4.4 for all the OS mass fractions (Fig. 1d).

The consequences of the interaction between pH and temperature on the inulinase activity are also shown in Fig. 1. Inulinase activity was about 15 U/mL at 25 and 70 %, while at 50 % it was about 28 U/mL (Fig. 1a). At pH=4.8, the values were 9 U/mL at 25 %, and 11 U/mL at 50 and 70 % of OS (Fig. 1b). The most appropriate OS mass fraction for free inulinase activity was 50 % at 55 °C and pH=4.8 (Figs. 1a and b). At low OS mass fraction, namely 25 %, water could cause enzyme agglomeration, increasing the mass transfer limitations. In addition, water could act as a competitive inhibitor for the substrates. At high OS mass fraction, namely 70 %, the enzyme activity was drastically reduced, due to increased enzyme rigidity, constituting a stable transition state (*14*).

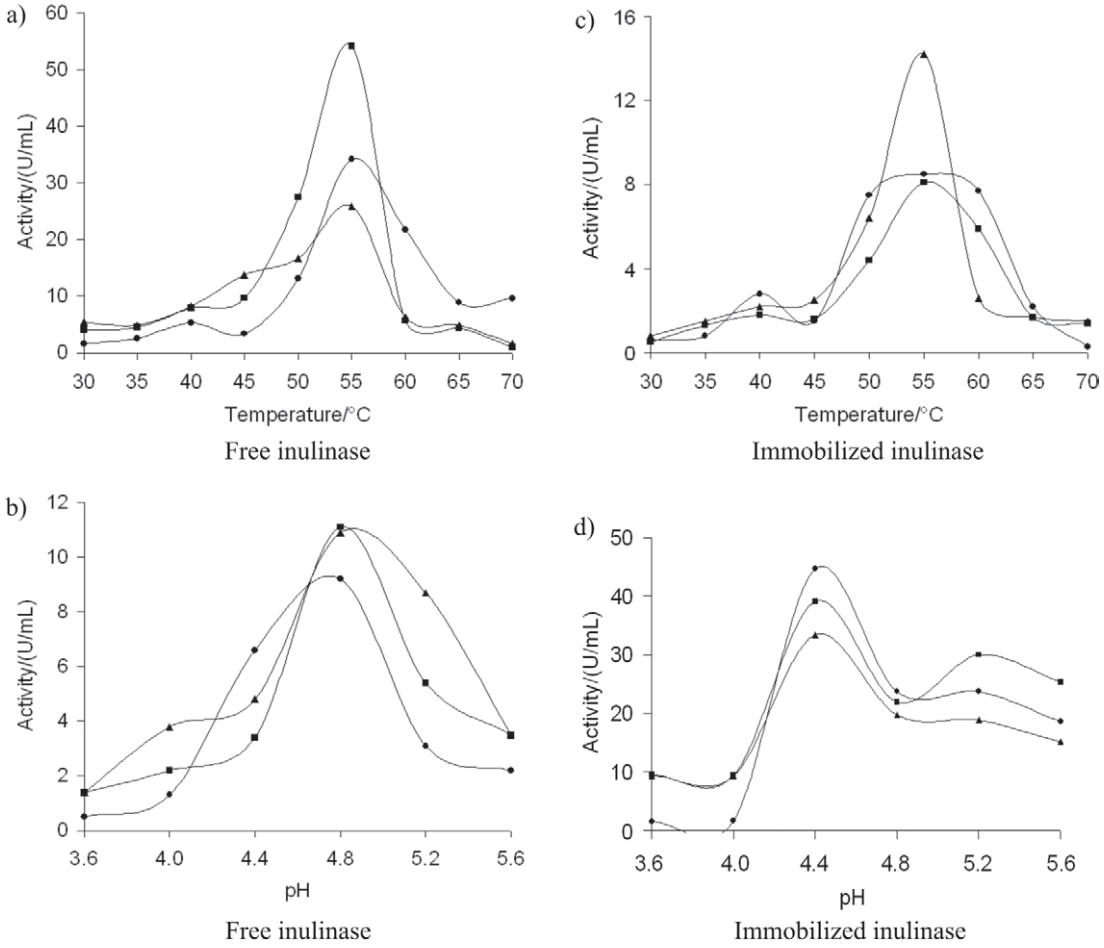

Fig. 1. The influence of (a and b) optimal temperature (30 to 70 °C) and (c and d) pH (4.0 to 5.6) on inulinase activity after 10 min of reaction at the three organic solvent mass fractions (● 25, ■ 50 and ▲ 70 %)

For the immobilized enzyme at pH=4.8, the activity was about 25 U/mL at 25 % (by mass) of OS, and under the optimum conditions (55 °C and pH=4.4), the activity was about 45.0 U/mL (Fig. 1d). In addition, the enzyme activity decreased with the increase in OS mass fraction, but this fact was related to the optimum temperature for the enzyme activity, since at 50 and 70 % of OS the most appropriate temperature was 55 °C, as can be seen in Fig. 1c. Analyzing Fig. 1d (50 °C and pH=4.4), the OS mass fraction showed little influence on enzyme activity, differently from that verified for the enzyme in the free form (Fig. 1a).

Analyzing Figs. 1a and c, referring to the optimal temperature for the free and immobilized inulinase, respectively, the free enzyme showed a catalytic power about 3 times greater than that of the immobilized enzyme at all OS mass fractions. This was because the optimal pH for the immobilized inulinase was 4.4 (Fig. 1d) and the experiments for the determination of optimal temperature were carried out at pH=4.8 (optimal pH for the free enzyme). These results suggest that small variations around the optimal point for enzyme activity cause distinct alterations in the catalytic power. For industrial use, the conditions required for the maximum reaction rate must be known, but this is not the only aspect to be considered, since the enzyme stability under those conditions should also be determined.

The optimal temperature and pH values for free and immobilized inulinase had previously been reported only for aqueous solutions. Paula *et al.* (*15*) found that the optimal pH was 3.5 in both cases, and the optimal temperatures were 55 and 60 °C for the free and immobilized inulinase from *Kluyveromyces marxianus* ATCC 16045, respectively. Santos *et al*. (*12*) showed that the inulinase from *Kluyveromyces marxianus* ATCC 16045 showed higher activities and stabilities at pH=4.4 and pH=4.8, and at temperatures of 63 and 57.5 °C, for the free and immobilized enzymes, respectively. Singh *et al*. (*16*) showed that the optimal pH was 5.5 in both cases, and the optimal temperatures 50 and 55 °C for the free and immobilized inulinase from *Kluyveromyces marxianus* YS-1, respectively. Catana *et al*. (*17*) found an optimal temperature of 55 °C for the free enzyme and in the range from 50 to 60 °C for the immobilized enzyme, and the optimal pH was 4.0 for the immobilized enzyme and 5.0 for the free enzyme. Kushi *et al*. (*18*) and Cazetta *et al*. (*19*) reported optimum temperatures of 55 and 60 °C for the free and immobilized inulinase, respectively, from *Kluyveromyces marxianus* ATCC 16045.

Effect of temperature on inulinase stability

Table 1 summarizes the data referring to the thermal stability of free and immobilized inulinase for the three butyl acetate mass fractions. As expected, the free enzyme showed the greatest stability at 50 °C, and the OS mass fraction had no significant effect on the stability. The stability of the free enzyme decreased sharply at temperatures higher than 55 °C, indicating that it had restricted application above this temperature. The temperature dependence of the inactivation rate constants (k_d) in the investigated temperature range could adequately be described by the Arrhenius equation (data not shown), yielding a deactivation energy (E_d) of about 351 kJ/mol at all the OS mass fractions. This value is much lower than that obtained by Santos *et al*. (*12*) (509 kJ/mol) in aqueous systems.

Analogous to the free enzyme, the greatest stability of the immobilized form was at 50 °C and the OS mass fraction did not affect the stability. However, the immobilized enzyme showed greater thermal stability than

Table 1. Effect of temperature on the model parameters for the free and immobilized enzyme

Temperature/°C	*w*(OS)/%					
	25		50		70	
	$k_d \times 10^3$/min	Half-life/min	$k_d \times 10^3$/min	Half-life/min	$k_d \times 10^3$/min	Half-life/min
	Free enzyme					
50.0	1.0	693.0	1.4	495.0	0.8	866.3
52.5	3.8	182.4	4.2	165.4	4.5	154.0
55.0	10.2	67.9	9.6	72.2	9.6	72.2
57.5	27.0	25.6	29.9	23.2	33.2	20.8
60.0	95.5	7.3	132.3	5.2	90.7	7.6
62.5	182.0	3.8	201.0	3.4	228.9	3.0
65.0	466.0	1.5	511.4	1.3	588.0	1.1
E_d/(kJ/mol)	352.0		352.0		356.0	
	Immobilized enzyme					
50.0	0.3	2310.3	0.35	1980.2	0.3	2310.3
52.5	1.2	577.6	1.4	495.0	1.3	533.1
55.0	2.7	256.7	2.7	256.6	3.0	231.0
57.5	5.9	117.4	5.3	130.7	6.7	103.4
60.0	12.6	55.0	10.3	67.3	14.8	46.8
62.5	26.7	25.9	19.7	35.1	32.2	21.5
65.0	0.3	2310.3	0.35	1980.2	0.3	2310.3
E_d/(kJ/mol)	282.0		245.0		293.0	

the free enzyme. As an example, the half-life of the immobilized enzyme at 62.5 °C (25 min) was similar to the value obtained for the free enzyme at 57.5 °C, and at 50 °C the immobilized enzyme presented a half-life about three times greater than that of the free inulinase. The mean deactivation energies (E_d) of about 273 kJ/mol obtained at all the OS mass fractions were in agreement with the data reported by Santos *et al*. (*12*) (298 kJ/mol) for immobilized inulinase in an aqueous system.

The application of the thermal parameters presented in Table 1 in order to predict the deactivation enzyme kinetics is shown in Fig. 2 for the temperatures of 50, 55 and 65 °C. As can be observed, the deactivation followed the first order kinetics, since the model (Eq. 1) was in agreement with the experimental data according to the changes in temperature for both free and immobilized inulinase.

The thermal stability of inulinase has been reported in the literature, and the data available are similar to those obtained in the present work. Paula *et al*. (*15*) reported that immobilized inulinase was more stable than the free enzyme. At 50 °C the free enzyme completely lost its activity after 24 h and the immobilized retained about 78 % of its activity. Similar behaviour was verified

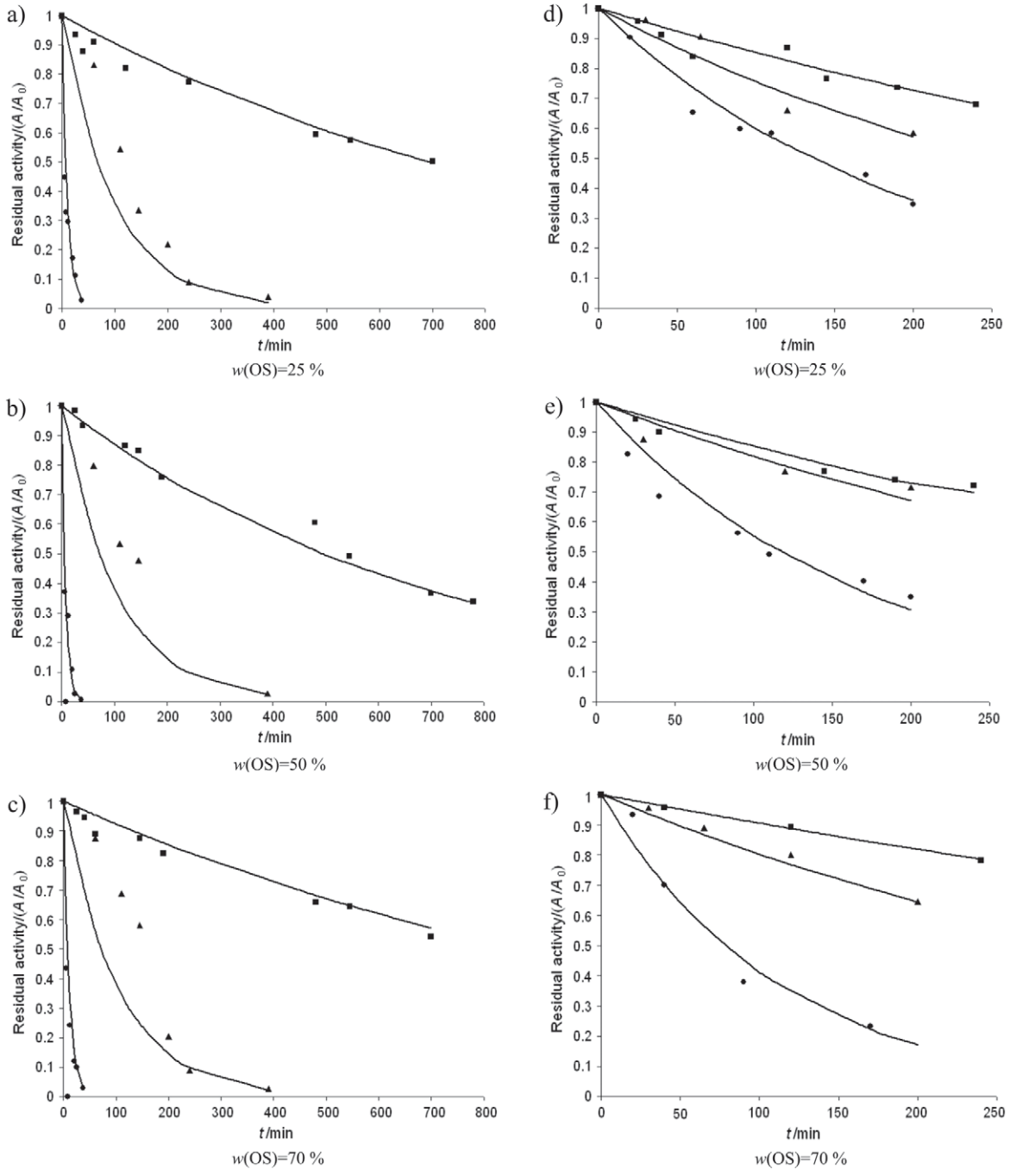

Fig. 2. Effect of temperature (● 50, ■ 55 and ▲ 60 °C) on (a, b and c) the stability of free and (d, e and f) immobilized inulinase at the three OS mass fractions. Experimental data and conditions are shown in Table 1

at 55 °C during the first 2 hours. Gill *et al*. (*20*) reported greater thermal stability, with residual activity of 70 % after 48 h at 55 °C for the immobilized enzyme from *Aspergillus fumigatus.* Singh *et al*. (*16*) reported that the immobilized inulinase retained more than 90 % of its original activity after incubation at 60 °C for 3 h, whereas the activity of the free enzyme was reduced to 10 %. At 70 °C, it was found to be 56 % active for up to 3 h, but was rapidly inactivated in the free form.

Effect of pH on inulinase stability

In this work, it was assumed that the pH inactivation of the enzyme followed the first-order kinetics. This assumption allows one to calculate the inactivation rate constants (k_d) and the half-life in a similar way to that employed in thermal inactivation. As can be seen in Fig. 3, the assumption adopted was valid, since the estimated parameters were in agreement with the experimental inactivation kinetics for the three different pH values, namely 4.4, 4.8 and 5.6.

Table 2 summarizes the effects of pH on the free and immobilized enzyme stability in terms of the half-life and inactivation rate constants for several pH values. The organic solvent mass fractions resulted in variations of the free enzyme stability. At 25 % (by mass) of OS, inulinase was more stable at pH=4.8, showing a half-life

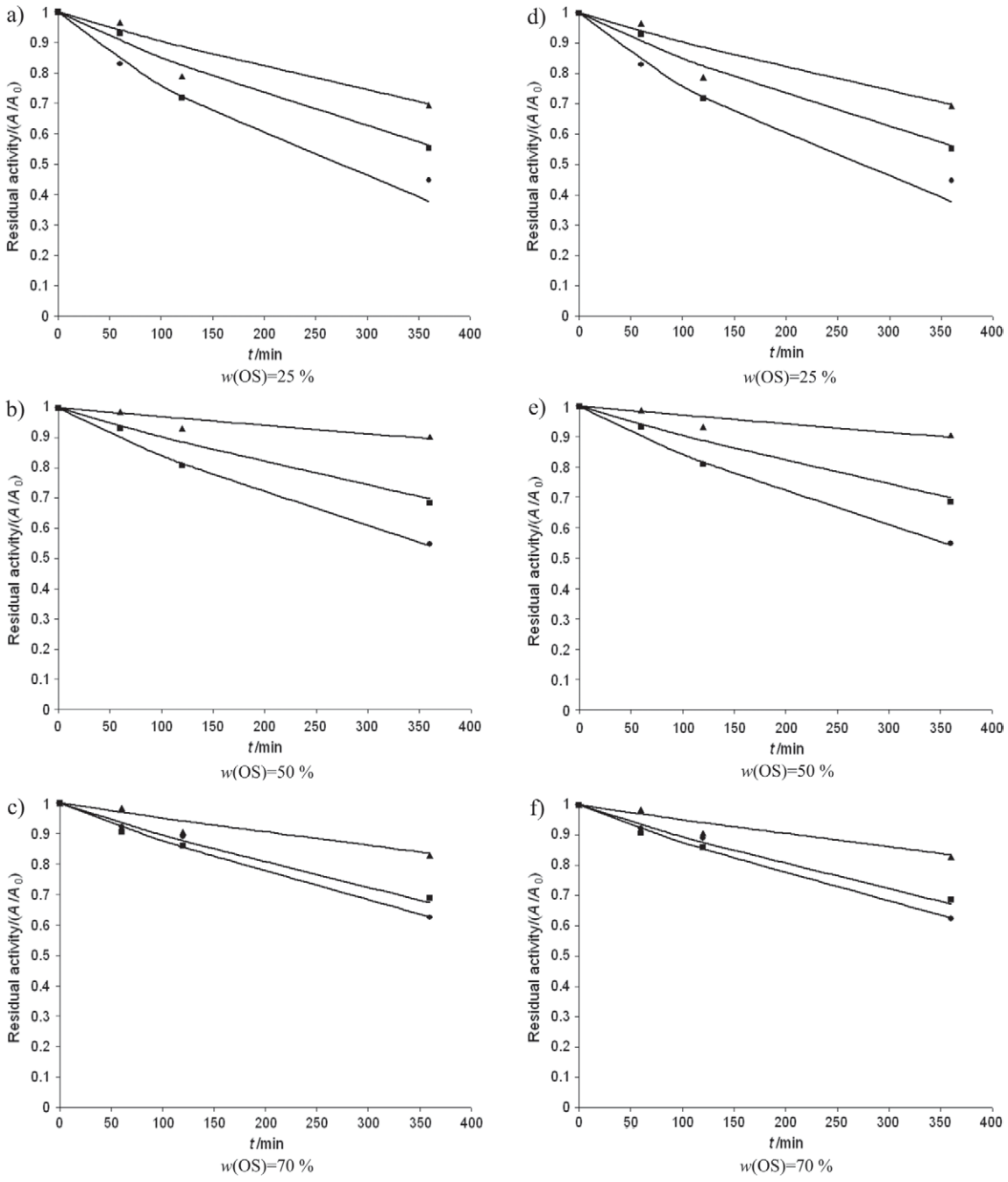

Fig. 3. Effect of pH (● 4.0, ■ 4.8 and ▲ 5.6) on (a, b and c) the free and (d, e and f) immobilized inulinase at three organic solvent mass fractions. Experimental data and conditions are shown in Table 2

Table 2. Effect of pH on the model parameters for the free and immobilized enzyme

pH	*w*(OS)/%					
	25		50		70	
	$k_d \times 10^3$/min	Half-life/min	$k_d \times 10^3$/min	Half-life/min	$k_d \times 10^3$/min	Half-life/min
			Free enzyme			
4.0	1.3	533.1	1.1	630.0	0.7	990.0
4.4	1.4	495.0	2.8	247.5	2.4	288.8
4.8	1.5	462.0	0.8	866.3	3.6	192.5
5.2	2.7	256.7	1.1	630.0	3.7	187.3
5.6	8.5	81.5	8.5	81.5	8.1	85.6
			Immobilized enzyme			
4.0	1.6	433.1	1.0	693.0	1.0	693.0
4.4	1.3	533.1	1.2	577.5	1.2	577.5
4.8	1.0	693.0	0.3	2310.0	0.5	1386.0
5.2	0.9	770.0	0.9	770.0	1.6	433.1
5.6	2.3	301.3	1.7	407.6	1.3	533.1

of 530 min. At 50 % of OS, it was more stable also at pH=4.8, with a half-life of 866 min. However, the most stable free enzyme was found at 70 % of OS and pH=4.0, when the half-life was 990 min.

Considering the immobilized enzyme, at 25 % of OS the inulinase was more stable at pH=5.2 (half-life of 770 min). At 50 and 70 % of OS the inulinase showed greater stability at pH=4.8, with half-lives of 2310 and 1386 min, respectively. In general terms, the immobilized enzyme was shown to be more stable in the whole pH range investigated in this work. As an example, at 70 % of OS, there was a drastic inactivation effect on the free enzyme at pH values above 4.0. For the immobilized enzyme, there was no such inactivation effect, and more importantly, the stability was improved by about 2.5 times as compared to the free enzyme.

Considering the effect of pH in the enzymatic reactions, although microbial inulinases are described as stable at pH values from 3.5 to 6.5 (*21*), in their research, Cazetta *et al*. (*19*) observed that the optimum pH for the free enzyme was 4.0, a subsequent decrease in activity occurring from this point, reaching 0 at pH=10.5. Similar values have been reported in the literature. Kushi *et al*. (*18*) and Ettalibi and Baratti (*22*) obtained maximum inulinase stability at pH=4.7 for the free enzyme obtained from *Kluyveromyces marxianus* var. *bulgaricus* and *Aspergillus ficum*, respectively. Singh *et al*. (*16*) studied the effects of pH on the stability of free and immobilized inulinase, and found that at pH=4.5 and pH=6.5, respectively, the immobilized enzyme retained more than 80 % of its initial activity after 6 h of incubation, whereas the free enzyme only retained 60 %.

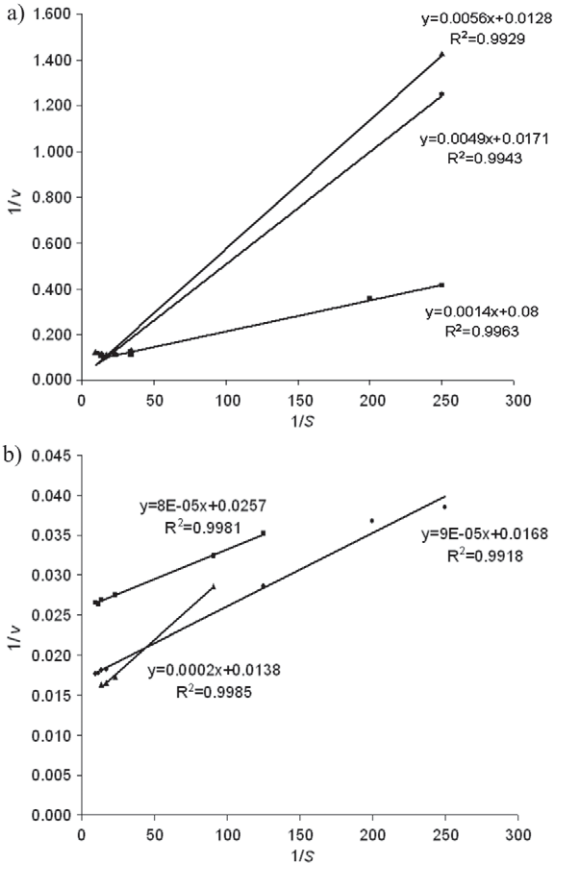

Fig. 4. Lineweaver-Burk plots for (a) free and (b) immobilized inulinase at different organic solvent mass fractions (● 25, ■ 50 and ▲ 70 %). Experimental data and conditions are shown in Table 3

Kinetic parameters of the free and immobilized inulinase

The kinetic constants v_{max} and K_m for the free and immobilized inulinase were calculated from the plots shown in Fig. 4 for all the OS mass fractions, and Table 3 summarizes the obtained results. For the free enzyme, an accentuated increase in the values of v_{max} as for K_m was verified as the butyl acetate mass fraction increased from 25 to 70 % (by mass). Apparently, the OS improved the maximum reaction rate, but this could be a compensatory effect, since the affinity of the enzyme for the substrate was drastically reduced at 50 and 70 % (by mass). In fact, for a reaction occurring at high K_m values, the maximum reaction rate would be too high, otherwise the reaction rate would give low values.

Table 3. Kinetic parameters for the free and immobilized inulinase at the three organic solvent (OS) mass fractions

w(OS)/%	25	50	70	25	50	70
	v_{max}/(U/mL)			K_m/mM		
Free	17.5	58.5	78.1	14.8	280.7	437.5
Immobilized	38.9	59.5	72.5	3.1	5.4	14.0

For the immobilized enzyme, v_{max} showed a similar behaviour to that reported for the free enzyme. The affinity between the enzyme and the substrate decreased with the increase in the butyl acetate mass fraction, since the K_m values increased. However, for the immobilized inulinase, the impact of the OS mass fraction on the K_m values was less accentuated. Such results for the immobilized enzyme can be considered satisfactory, since high v_{max} and low K_m values lead to a high reaction rate. However, it is important to remember that for the immobilized enzyme, K_m and v_{max} are apparent parameters, because the mass transfer resistances should be considered in the analysis, since they may be the limiting step in the process.

By comparison, the immobilized inulinase from *Kluyveromyces* genus showed K_m values in the range from 7.7 to 10 mM for *K. fragilis* (*21*) and 13.3 mM for *K. marxianus* (*18*). The results obtained in the present work for the immobilized enzyme are in agreement with the previously cited references. However, K_m values are considerably lower than those obtained by Paula *et al*. (*15*) using the same strain to produce inulinase, but the experiments were carried out in citrate buffer and the immobilization method was not the same.

Conclusions

In the present work, the effect of butyl acetate mass fraction on the characteristics of free and immobilized inulinase was evaluated. Neither the free nor the immobilized enzymes were inactivated by the organic solvent in the whole investigated range, indicating that inulinase could be used for synthesis in an organic medium. This aspect is favourable for the industrial application of this enzyme since its use is not restricted to aqueous solutions, allowing for exploratory studies of its catalytic power in other solvents. It was shown that immobilization improved enzyme stability. Regarding the temperature, the immobilized enzyme was about 3 times more stable than the free enzyme at 50 °C, while regarding the pH, the immobilized enzyme showed the greatest stability at 50 % (by mass) of OS and pH=4.8.

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