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CHARACTERIZATION OF SILICA-BASED BIOMATERIALS CONTAINING MICROBIAL AMYLASES

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Amylases are one of the most important industrial enzymes. Large arrays of amylases are involved in the complete breakdown of starch. Amylases are of ubiquitous occurrence; however, microbial sources are the most preferred ones for large-scale production. Today a large number of microbial amylases are marketed with applications in different industrial sectors, for example in ethanol and sweetener industry. In the past decade, the encapsulation of enzymes inside inorganic sol-gel matrices has become a generic method to prepare efficient biocatalysts that are easy to recycle. The aim of this work was to study the influence of several parameters on the activity and stability of immobilized bacterial α-amylase, in xerogel. Silica sols were obtained using the following precursors: tetraethoxysilane (TEOS), methyltriethoxysilane (MTES), phenyltriethoxysilane (PhTES). The pH and temperature profiles and the kinetic parameters (K_M, V_{max}) were determined, in comparison with the native enzyme.

INTRODUCTION

Amylases are enzymes that hydrolyze the starch molecules into polymers composed of glucose units. Widespread studies of amylolytic enzymes have primarily been related to their application in industries, such as in food, detergents, textiles and paper industry. The spectrum of amylase applications has expanded into many other fields, such as clinical, medicinal and analytical chemistry. [1, 2, 3] In the light of modern biotechnology, the immobilization of microbial amylase by sol-gel method is gaining an increasing interest. Entrapped enzymes offer many advantages over the native one due to the ease of their reuse and recovery. The sol-gel method of immobilization offers several advantages including biocompatibility, resistance to the microbial attack, thermal and chemical stability of the matrix, low processing temperature, readily control of porosity. [4, 5]

The aim of this work was to study the influence of several parameters on the activity and stability of immobilized bacterial α-amylase, in xerogel. Silica sols were obtained using the following precursors: tetraethoxysilane (TEOS), methyltriethoxysilane (MTES), phenyltriethoxysilane (PhTES). The pH and temperature profiles and the kinetic parameters (K_M, V_{max}) were determined, in comparison with the native enzyme.

RESULTS AND DISCUSSION

α-Amylase was immobilized in gels derived from tetraethoxysilane (TEOS) and organosilanes (i.e. methyltriethoxysilane (MTES), phenyltriethoxysilane (PhTES)) in different molar ratios: PhTES:TEOS 1:1 and 2:1, MTES:TEOS 1:1, 2:1 and 3:1. To find the optimal parameters for the immobilized enzyme the effects of the pH and temperature were investigated.

The effect of the pH on the activities of the native and immobilized enzyme was investigated in the pH range of 2.6 – 8 at room temperature. The maximum studied pH value was 8 because in aqueous solution silica solubility increases rapidly

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at more basic pH. This can cause the dissolution of the gels, leading to a decrease in the surface area and an increase in the pore size. [6] As it is showed in Fig. 1 the native enzyme has a characteristic pH profile, the classic "bell-shaped" curve. Regardless the type of matrix, the immobilized enzymes behave quite similar. In the pH range 2.6 - 3.6 and 6.6 - 8 the activities of the entrapped enzyme were much higher than its free form. The optimal pH of the immobilized enzymes was shifted towards the alkaline side (with approximately two pH units) when compared with that of the native enzyme. This shift is a result of the immobilization process. The sol-gel matrices have a porous structure and can form plenty of hydrogen bonds with the enzyme that change its surface charge distribution. Also, the immobilized enzyme better tolerance to the extreme pH conditions is presumably owing to the macromolecular additive, PVA, that protect the enzyme from denaturing.

We have also investigated the dependence of the native and immobilized enzyme activities on temperature in a range of 20-90°C. The optimal temperature for attaining highest activities was 60°C for the native enzyme and between 30 and 45°C for the immobilized ones, depending on the type of matrix (Fig. 2). The native enzyme presents a characteristic ("bell-shaped curved") temperature profile. By immobilization, the optimal temperature for the immobilized enzyme was shifted towards smaller values. In the case of PhTES:TEOS matrices a good thermal stabilization was obtained, (over 85% residual activity till 90°C). The enzyme immobilized in MTES:TEOS matrices presents also a good stabilization but only till around 60°C. The native enzyme activity presents a steep decrease below both 40°C and over 80°C. However, the relative activities of the immobilized enzyme were much higher than that of the native one in the temperature range 20 - 45°C. The high residual activities at low temperature (20-45°C) make possible the efficient technological use of immobilized enzyme at medium temperature. Overall, the sol-gel matrices have a protective effect, stabilizing the enzyme activity over a wider temperature range, but also shift the optimal temperature to the low values. PhTES:TEOS matrices (the most hydrophobic) have the best protective effect, greater than MTES:TEOS and TEOS matrices, probably because of their increased hydrophobicity.

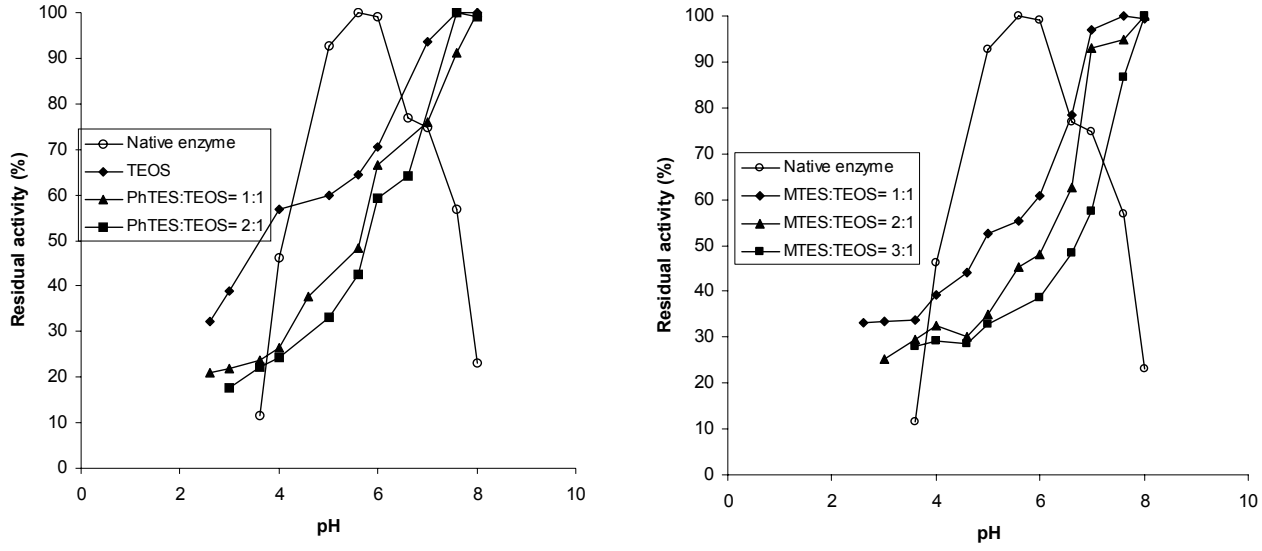

Fig. 1 – The effect of the pH on activity of immobilized and native α-amylase.

The saturation curves of the initial rates of amylase obtained from experimental data are presented in Fig. 3. The kinetic parameters of the enzymatic reaction estimated by fitting the experimental data to the Michaelis-Menten equation are presented in Table 1. The Michaelis-Menten constants K_M and V_{max} for the native enzyme were 135.6 mg/mL and 1.44 mg/mL·min, respectively. Both the K_M and the V_{max} values seemed to be affected by the immobilization process. Regardless the types of matrix, V_{max} values are lower than that of the native enzyme probably due to the diffusion of substrate from the bulk solution to the microenvironment of immobilized enzyme that can limit the rate of the enzyme reaction. The low K_M reflects a higher

affinity to substrate. The high affinity of the enzyme to the substrate may be explained by the fact that, when binding onto the surface of the silica particles, the enzyme rearranged itself to present a better conformation. This rearrangement in conformation may result in better availability of its active sites. A more hydrophobic matrix may enhance the enzyme-substrate affinity. The highest K_M values were obtained in the case of PhTES:TEOS matrices and the lowest for TEOS. For the same type of matrix, by increasing the amount of the hydrophobic precursor, the K_M value increases and the catalytic efficiency decreases. The V_{max}/K_M ratio shows that the catalytic efficiency is two times enhanced by immobilization in sol-gel matrices, obtained from TEOS. The catalytic eficiency is decreasing with the increasing hydrophobicity of the precursors. In conclusion, the hydrophobic matrices are less favorable for the amylases, unlike for lipases, which prefer a more hydrophobic medium. [6] The optimum sol-gel precursor for amylase immobilization seems to be TEOS.

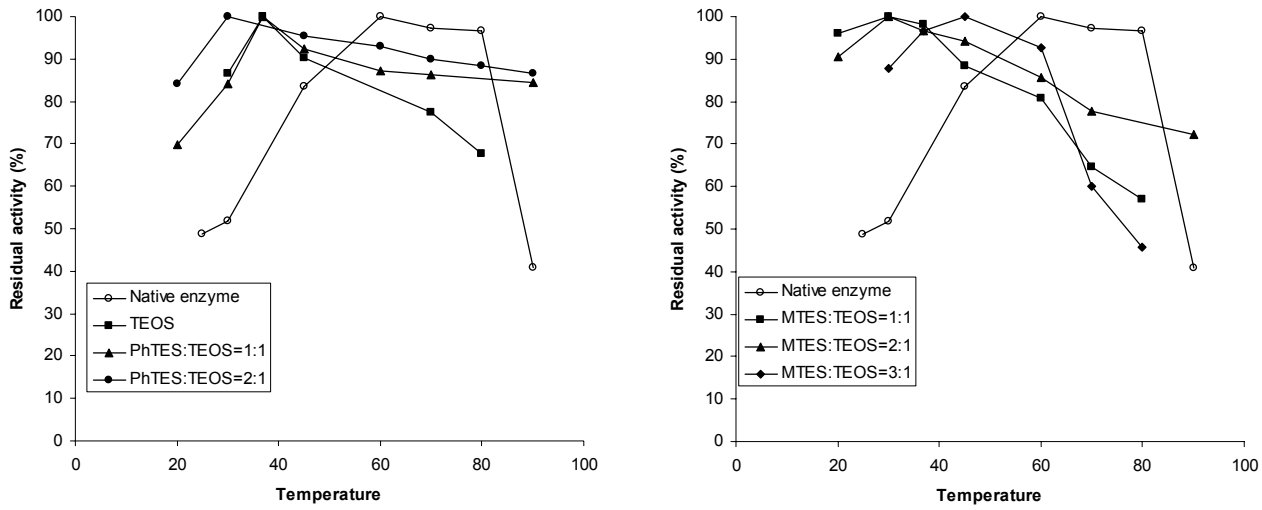

Fig. 2 – Temperature effect on activity of immobilized and native α-amylase.

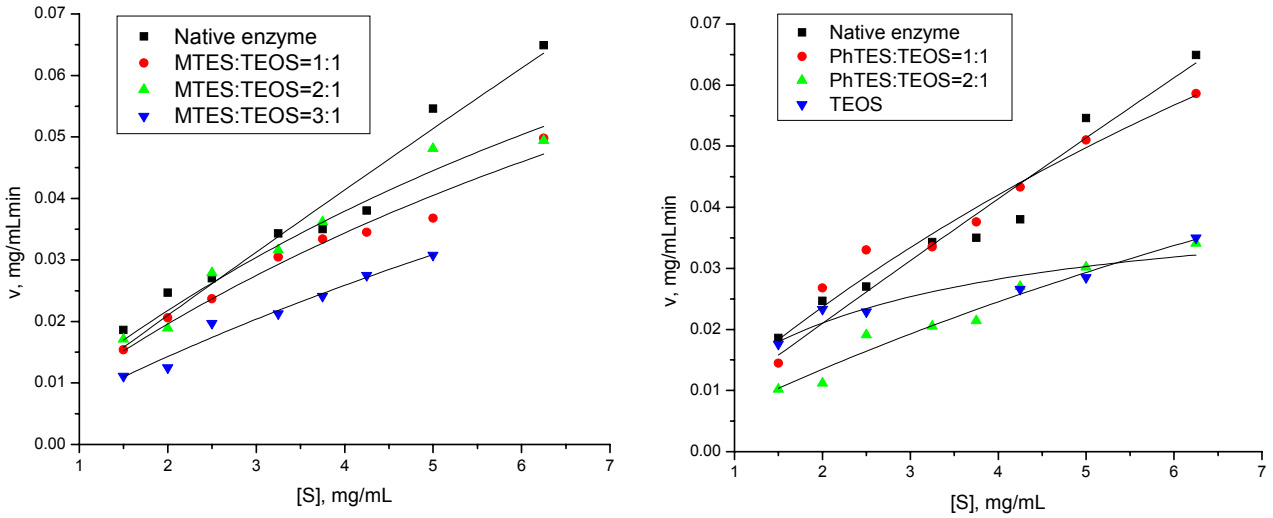

Fig. 3 – Initial rates (v) vs. substrate concentration ([S]) plots (saturation curves) of native and immobilized α-amylase.

Table 1

Kinetic parameters for the native and immobilized α-amylase, estimated by fitting the experimental data to the Michaelis-Menten equation

Matrix obtained by one step method of immobilization	V_{max} mg/mL·min	K_M mg/mL	$V_{max} \cdot 100 / K_M$, min^{-1}
-	1.44	135.66	1.06
TEOS	0.04	2.07	1.93
PhTES:TEOS 1:1	0.18	13.85	1.30
PhTES:TEOS 2:1	0.14	18.08	0.77
MTES:TEOS 1:1	0.14	12.31	1.14
MTES:TEOS 2:1	0.15	12.91	1.16
MTES:TEOS 3:1	0.14	17.14	0.82

EXPERIMENTAL

Encapsulation of enzyme by sol-gel process

The sols were prepared using the following Si-precursors: tetraethoxysilane (TEOS), methyltriethoxysilane (MTES) and phenyltriethoxysilane (PhTES). The molar ratios are PhTES:TEOS 1:1, PhTES:TEOS 2:1, MTES:TEOS 1:1, MTES:TEOS 2:1, MTES:TEOS 3:1. The mixture of alkoxides in different molar ratios and enzymatic solution containing 18.75 mg α-amylase (1:1.7, v/v) were mixed with PVA 22.000 (polyvinyl alcohol 22.000) 4%, NaF 1 M and isopropylic alcohol (2:1:2, v/v). The gels were left overnight for aging (4ºC), washed with n-hexane and dried (4ºC). [7, 8]

Residual starch concentration assay (I_2/I^-): 0.5 mL soluble starch (0.4%), 0.4 mL phosphate buffer (0.05 M, pH 5.2) and 0.01 g immobilized biocatalyst were kept for 5 min. at 25ºC. 5 mL solution I_2/I^- M/1000 and 15 mL distilled water were added. The samples were filtered. The absorbance was measured at 595 nm against distilled water. One unit of amylase activity was defined as the amount of enzyme required to hydrolyze 1 mg starch in 5 min at 25°C when 2 mg starch was present at the start of the reaction. [9]

The effect of the temperature on the activity of native and immobilized enzyme was estimated by residual starch concentration assay at various temperatures. The test tubes were stored in a water bath at specific temperature (20, 30, 37, 45, 60, 70, 80 and 90°C).

The effect of the pH on the activity of native and entrapped enzyme was investigated by residual starch concentration assay, but in the presence of citric acid 0.1 M – Na_2HPO_4 0.2 M buffer ranged from pH 2.6 to 8 at room temperature.

Determination of kinetics parameters of native and immobilized enzyme: K_M and V_{max} were determined by measuring initial rates of Zulkowsky starch hydrolysis. Kinetics studies were conducted in citrate –phosphate buffer 0.15 M, pH 4.6, at 37°C in a 50 mL stirred jacketed batch reactor. The starch concentrations were 1.5, 2, 2.5, 3.25, 3.75, 4.25, 5 and 6.25 mg/mL. The reaction was started by addition of the enzyme (4 mL native enzyme and 20 mg immobilized enzyme) and 1 mL samples were collected every 2 minutes during the first 20 minutes of the reaction. The residual starch concentration assay was used to analyze the samples. [10] The values of K_M and maximal velocity (V_{max}) were calculated by adjusting the experimental data from the saturation curve to the Michaelis-Menten equation by a computer aided nonlinear regression analysis.

CONCLUSIONS

Optimum pH of the immobilized enzyme was shifted to basic side by two units when compared to the optimum pH of the native enzyme (5.6). Optimum temperature of the immobilized enzyme was 30°C and 45°C, depending on the type of matrix, decreased by 30°C and 15°C, respectively when compared to optimum temperature of the native enzyme (60°C). Compared to the free form, the immobilized enzyme exhibited improved thermal and pH stability.

Kinetic parameters values were lower than those of the native enzyme. The rate of the enzymatic reaction is limited probably due to diffusional resistance of the carrier against substrate and/or products. The low K_M values reflect the high affinity to substrate possibly due to the rearrangement in conformation of the enzyme when binding onto the surface of the silica particles.

The hydrophobic matrices are less favorable for the amylases, unlike for lipases, which prefer a more hydrophobic medium. The optimum precursor for amylase probably is TEOS.

These preliminary results suggest that the α-amylase immobilized in silica gel is a robust and relatively stable biocatalyst, which maintains its catalytic activity and can be used in various biotransformations.

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