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Immobilization of *Aspergillus niger* Pectinase on Polyacrylonitrile Copolymer Membrane

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Abstract: *Aspergillus niger* pectinase was successively immobilized on polyacrylonitrile copolymer membrane. The optimal duration of immobilization process and optimal enzyme concentration of 1% (w/v) at pH 3.0 were determined. Immobilized enzyme showed a shift in pH optimum which was at pH 5.0 for the enzyme immobilized on not activated membrane and at pH 5.5 for the enzyme immobilized on membrane activated with glutaraldehyde. Both immobilized enzymes exhibited maximum activity at 60°C. Free pectinase exhibited higher stability than that of the immobilized enzyme at 30, 40 and 50°C. In order to examine the reusability of immobilized pectinase 22 consecutive batch cycles were performed.

Key words: Acrylonitrile membrane, *Aspergillus niger*, immobilization, pectin, pectinase

INTRODUCTION

Pectinolytic enzymes are a group of enzymes which hydrolyze pectin, one of the main polysaccharides in plant cell wall. The primary chain of pectin is composed of α-1,4- linked residues of D-galacturonic acid (Jayani *et al*., 2005). The enzymes depolymerising pectin can be divided into hydrolases and lyases. Hydrolases degrade pectin by hydrolysis of α-1,4-glycosidic linkages in the polymer chain. Lyases cause depolimerisation by transelimination which results in formation of unsaturated bond between C_4 and C_5 at the nonreducing end (Sakai *et al*., 1993). Pectinases are distributed in many higher plants and microorganisms. They play a very important role in plants since they help in cell wall extension and softening of some plant tissues (Jayani *et al*., 2005).

Pectinases find commercial application in production of fruit juices and wines, mainly for juice clarification and for improving pressing and extraction of the juice from fruits and vegetables (Kashyap *et al*., 2001). The raw fruit juice obtained after pressing is very turbid, viscous and contains a significant amount of colloidal compounds, mainly pectin which cause cloudiness. The amount of colloids present in fruit juices is in the range of 100-1000 mg L^{-1}. Clarification involves the removal of juice haze by enzyme hydrolysis with pectolytic enzymes. After its degradation pectin- protein complexes flocculate giving a juice with lower viscosity which is easier to filter (Alvarez *et al*., 1998, 2000).

Microbial pectinases account for 25% of the global food enzymes sales. Most commonly used in industry are pectinases from *Aspergillus niger* which produces a mixture of polygalacturonase, pectinlyase and pectinesterase (Jayani *et al*., 2005; Almeida *et al*., 2005).

It is well known that immobilization of enzymes offers several advantages that include reuse of the enzyme and its easier separation from the product. Since in food industry it is preferably to avoid the presence of extraneous compounds in the final products the possibility to remove the enzyme is

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a significant advantage. In literature there is data about immobilization of pectinolytic enzymes on different supports by various of methods (Demirel *et al*., 2004; Vaillant *et al*., 2000; Rao *et al*., 2000; Sarioglu *et al*., 2001; Sardar and Gupta, 2005).

In the present study commercial pectinase from *Aspergillus niger* was immobilized on polyacrylonitrile copolymer membrane via adsorption on the membrane or covalently after activation of the support with glutaraldehyde. The methods used for immobilization are simple and effective. The chosen support has suitable pore size that allows easy penetration of the enzyme; it has high mechanical, temperature and chemical stability and can be separated from the reaction mixture without contaminating the final product.

MATERIALS AND METHODS

Commercial pectinase from *Aspergillus niger* (Biovet, Bulgaria), Apple pectin, 70% esterification degree, Polyacrylonitrile copolymer UF membrane (PAN) (Ekofilter, Bulgaria), 25 kDa.

The research was done in the department of Biochemistry and Molecular Biology, University of Food Technologies, Plovdiv, Bulgaria, in the period January- July, 2006.

Activation of the Membrane

The membrane was hydrolyzed with 6% NaOH for 60 min, then activated with 25% glutaraldehyde (GA) solution in water for 90 min (Godjevargova *et al*., 1994). After the activation the membrane was washed thoroughly until no GA is detected in the washings by measuring the absorbance at 235 nm.

Immobilization of Pectinase on PAN Membrane

The activated with GA and not activated membrane (20 cm^2) was immersed in 10 cm^3 enzyme solution and mild stirred at room temperature for 16 h. After the immobilization the membrane was washed thoroughly with distilled water, 0.5 M NaCl and C - P buffer until no protein is detected in the washings.

Determination of Enzyme Activity

Pectinase activity was determined by the method of Roboz (1952) by following the decrease in viscosity of 1% pectin solution in C-P buffer, pH 5.0, with a capillary viscometer at 30°C.

The percent decrease of viscosity of pectin solution is calculated by the equation:

$$\frac{t_o - t}{t_o - t_s} = \text{viscosity decreas (\%)}$$

where t_o- flow time of the substrate solution; t - flow time after the enzymatic reaction; t_s- flow time of the solvent.

RESULTS AND DISCUSSION

Commercial pectinase was immobilized on PAN membrane which is a good and used matrix for immobilization of enzymes (Godjevargova, 2004). For determining the influence of immobilization conditions only not activated membrane was used. The duration of immobilization is shown in Fig. 1.

The activity of the immobilized enzyme increased gradually till the eight hour and after that remained unchanged after 48 h immobilization time.

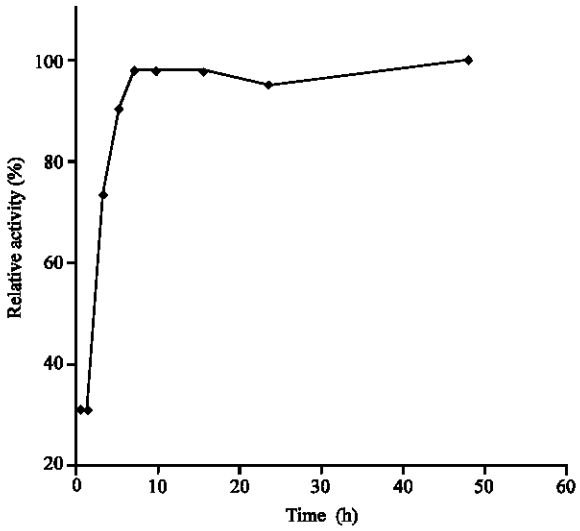

Fig. 1: Effect of duration of the immobilization process on the activity of immobilized pectinase

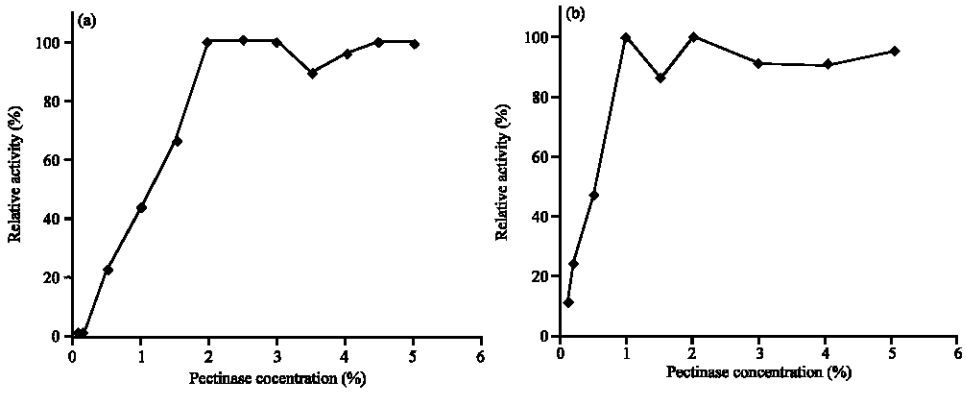

Fig. 2: Effect of pectinase concentration on the activity of the immobilized enzyme: (a) at pH 5.0, room temperature and (b) at pH 3.0, room temperature

The effect of pectinase concentration added for immobilization was studied. Starting from 0.1% (w/v) the activity of the immobilized enzyme raised to a maximal level. The optimal enzyme concentration was found to be 2% (w/v) at pH of immobilization 5.0 and 1% (w/v) at pH of immobilization 3.0 (Fig. 2a and b). For further experiments the second concentration of pectinase was chosen.

It can be seen that the effect of pH was well expressed and the immobilized enzyme had maximal activity at pH 3.0 of immobilization at 10°C, (Fig. 3) room temperature and 30°C. Above this pH the activity of immobilized Pectinase decreased and at pH of immobilization 6.0 and 7.0 was below 5%.

Immobilization changed the pH optimum of the enzyme which was at pH 4.0 for free pectinase (Fig. 4). The enzyme immobilized on not activated membrane exhibited optimal pH at 5.0 and the enzyme immobilized on activated with GA membrane at pH 5.5. This is in agreement with the results observed by Pifferi *et al*. (1988) for immobilization of endo-polygalacturonase and can be explained with a change of the microenvironment of the enzyme as a result of its immobilization to the matrix

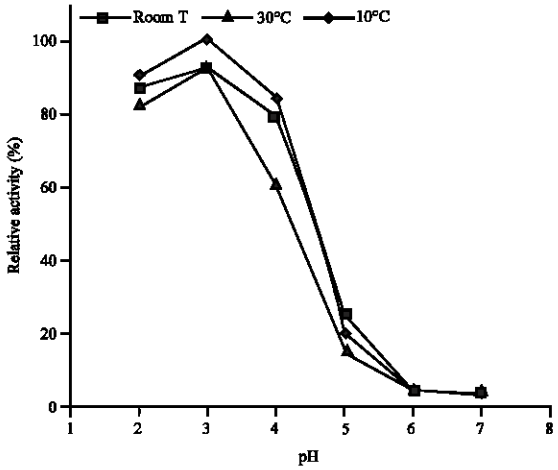

Fig. 3: Effect of pH and temperature of immobilization on the activity of immobilized pectinase

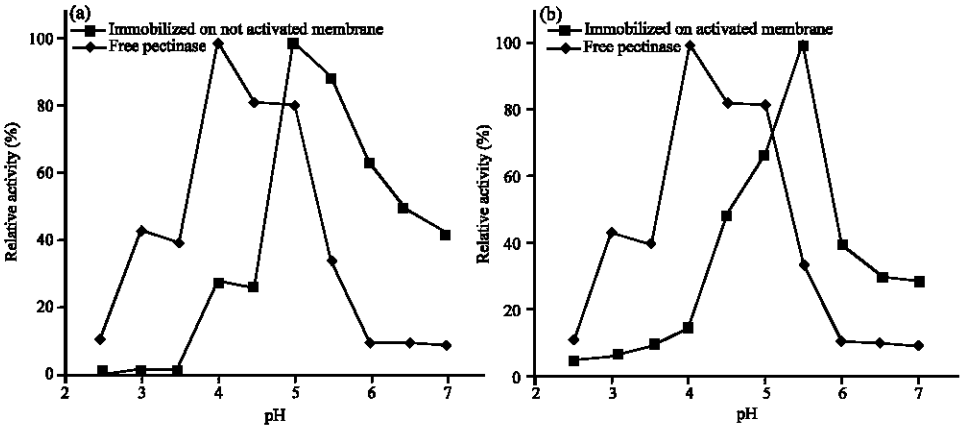

Fig. 4: Effect of pH on the activity of free and immobilized pectinase: (a) on not activated membrane and (b) on activated with GA membrane

although the chosen support is electrically neutral (Chibata, 1978). A shift in the pH optimum was observed also by Rexova- Benkova *et al*. (1986) in immobilization of endopolygalacturonase on ceramic materials and also by Vaillant *et al*. (2000) in immobilization of another pectinolytic enzyme pectin lyase on chitin and nylon. Except the change in the pH optimum there was also a change in the pH profile of the immobilized enzyme compared with the free form. Immobilized pectinase exhibited lower activity at pH below 4 than free enzyme and higher activity at pH above 5.0. The enzyme immobilized on not activated membrane retained more than 40% of its optimal activity at pH range 6.0-7.0 while free pectinase retained below 10% of its activity.

Free enzyme exhibited maximum activity at 50°C while the temperature optimum of immobilized on activated and not activated membrane enzyme elevated to 60°C (Fig. 5). As a result from enzyme binding to the matrix by covalent or non covalent bonds the rigidity of enzyme molecules is increased so that the protein is less affected by the denaturing effect of temperature (Spagna *et al*., 1997).

At 30, 40 and 50°C the stability of free pectinase was greater than that of the immobilized one (Fig. 6). At 30°C free and immobilized enzyme on activated with GA membrane retained its maximal

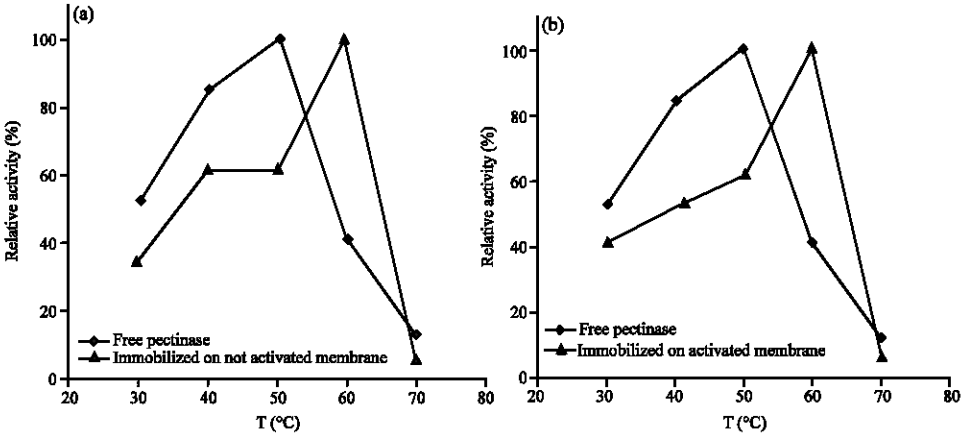

Fig. 5: Effect of temperature on the activity of free and immobilized pectinase: A- on not activated membrane; b- on activated with GA membrane

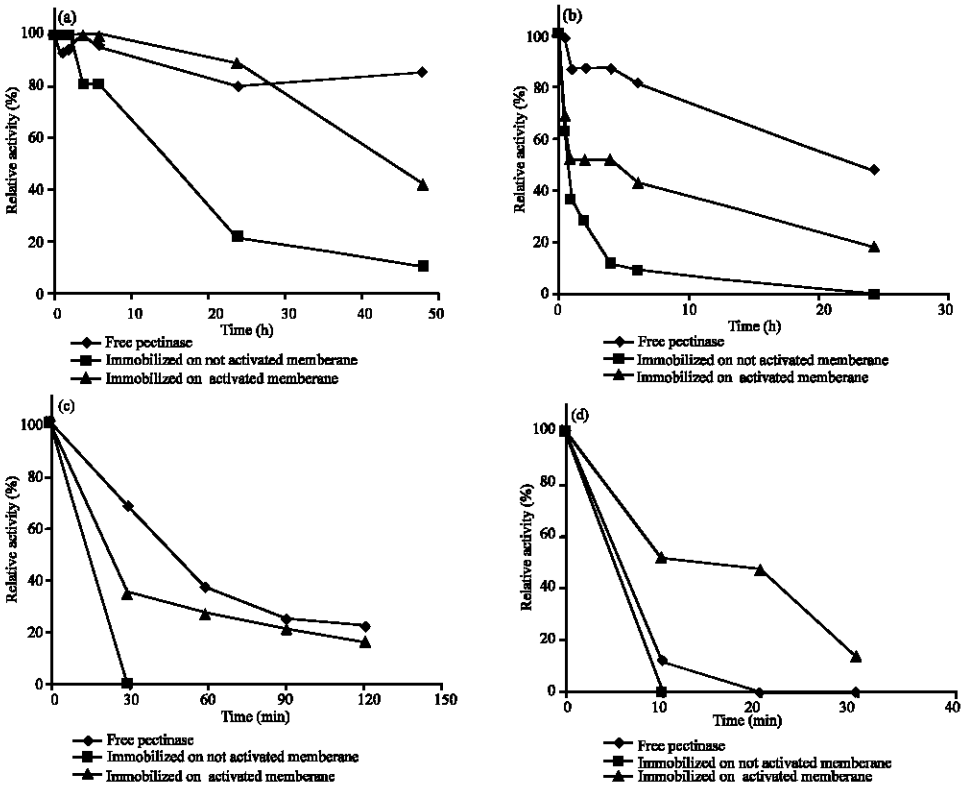

Fig. 6: Thermal stability of free and immobilized pectinase at pH 4.0: (a) -30°C; (b) -40°C; (c) -50°C; (d) -60°C

activity for 6 h incubation and the enzyme immobilized on not activated membrane only for 2 h. At 40°C pectinase immobilized on not activated PAN membrane almost lost activity for 6 h. For the same

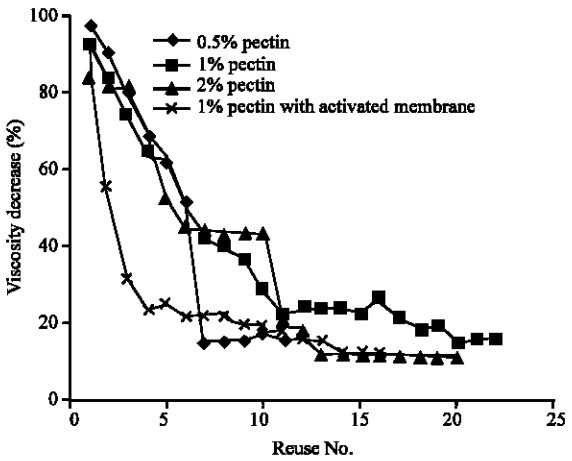

Fig. 7: Reusability of immobilized pectinase

time free pectinase retained 80% of its original activity while the enzyme immobilized on activated membrane retained 43%. At 50°C pectinase immobilized on not activated membrane completely lost activity for 30 min while free and immobilized on activated membrane enzyme were active after 2 h incubation at this temperature. At 60°C pectinase immobilized on not activated membrane deactivated for 10 min while the immobilized on activated membrane retained 50% of its original activity after 20 min incubation. It is generally considered that immobilized enzymes exhibited higher thermal stability than that of the free ones. In this research it can be seen that free pectinase was more stable than pectinase immobilized on PAN membrane via adsorption on the membrane or via covalent binding. Probably this is due to weak bonds formed between the enzyme and the support that cause easy separation of the enzyme from the matrix. The enzyme immobilized on activated with GA membrane exhibited higher stability than that immobilized on not activated membrane. Obviously the covalent bonds formed between CHO- groups of the crosslinker and NH_2- groups of the enzyme retained the protein stronger to the support. The usability of immobilized pectinase in presented in Fig. 7. It was able to hydrolyze pectin in 22 consecutive batch cycles. After each cycle the membrane with immobilized enzyme was washed with distilled water and incubated with fresh portion of substrate. Activity is expressed as percentage of decrease in viscosity of pectin solution. Three different pectin concentrations were used for this experiment -0.5, 1 and 2%. Gradually after each new reuse the immobilized pectinase lost activity which is expressed as lower percentage viscosity decrease of pectin solution.

For first time *Aspergillus niger* pectinase was successively immobilized on polyacrylonitrile copolymer UF membrane activated with GA and not activated. The immobilization parameters were found- duration of immobilization, pectinase concentration, pH and temperature of the enzyme solution during the immobilization process. The effect of pH and temperature on the activity of free and immobilized enzyme and the stability at 30, 40, 50 and 60°C were determined. The reusability of immobilized pectinase in subsequent batch cycles was examined using apple pectin as substrate. The immobilized pectinase preparation can be used in practice for clarifying of fruit juices and wine. The chosen support (ultrafiltration membrane) offers the opportunity the process to be held in a membrane reactor in which enzyme treatment and filtration can be done in one step.

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