On the Possibilities of Immobilization and Utilization of Some Cellulase Enzymes

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Synopsis

Cellulase enzymes produced by the microorganisms *Aspergillus niger* and *Trichoderma viride* have been immobilized on Ponilex type ion exchangers, both by ionic and by covalent bonding. Immobilization efficiency has been determined for enzyme preparations obtained, by their activity, as well as by that of the resulted effluent, on using as substrate carboxymethyl cellulose, cellobiose, and filter paper. The catalytic activity of the immobilized products has been tested against solutions of carboxymethyl cellulose and a series of hemicellulose containing alkaline extracts resulted from the alkaline pretreatments of some vegetal materials. The presence of polyphenol compounds in the latter ones determines a series of peculiarities in the behavior of the enzyme preparations, such as the transformation of the hydrolysis products and reduction of the catalytic activity of the enzyme, after a certain period of reaction.

INTRODUCTION

The transformation of the natural macromolecular compounds in products capable of being assimilated by microorganisms is achieved by means of exocell specific enzymes. Usually, as depending on the microorganism used, these biocatalysts are released in the external medium in the form of complex mixtures, with a view to perform thus the degradation of all vegetal material component.[1] That is why, in order to give a practical significance to naturally occurring processes, information is needed on synthesis and accumulation of such enzymes, their characteristics and mode of action, the existing possibilities for their utilization, and recovery in various types of reactions. The greatest part of the researchers agree that an important share of the expenses involving practical application of various biotechnologies based on enzyme utilization is represented by their making.[2] On the other side, a specific and very important action is to be recorded when having the enzymes in a purified form, corresponding to the sequence of reactions that are to be performed. Consequently, one can appreciate that, at present, the problem of utilization and recovery of exocell enzymes from the culture broth of certain microorganisms becomes capital, especially in the case of biocatalysts involved in the transformation of the components existing in the vegetal biomass.[3]

In a previous paper of ours,[4] we have shown the possibility of immobilizing cellulase enzymes produced by *Aspergillus niger* on carriers of the acrylic anion exchanger type (Ponilex AS). The favorable results obtained induced us to extend our studies for enzymes synthesized by *Aspergillus niger* and also for those belonging to the *Trichoderma viride* microorganism, which were

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retained on Ponilex type ion exchangers, by covalent and ionic bonding. At the same time, with a view to occurring certain synergistic effects, the possibility of obtaining some active preparations by using complex enzymes mixtures produced by different microorganisms (*Trichoderma viride* and *Aspergillus niger*) has been tested. Immobilization efficiency has been determined both for enzyme preparations and for separated effluents on using carboxymethyl cellulose, cellobiose, and filter paper as substrates. The reaction capacity of the enzyme preparations has been tested against solutions of carboxymethyl cellulose and hemicellulose present in a series of alkaline extracts resulted from the pretreatment of some vegetal materials.

The obtained results are being analyzed in the present paper, along with the peculiarities met in the functioning and stability of the enzyme preparations.

EXPERIMENTAL

Enzyme Production. Liquids possessing enzyme activity have resulted from cultures of *Aspergillus niger* and *Trichoderma viride* microorganisms, on media having the following composition: KH_2PO_4 0.6 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $CaCl_2$ 7 g; thiamine 0.1 g; $(NH_4)_2HPO_4$ 1.0 g; microelemental solution ($ZnSO_4 \cdot 7H_2O$ 1.4 g/L; $MnSO_4 \cdot 4H_2O$ 1.6 g/L; $CoCl_2 \cdot 6H_2O$ 2.0 g/L; $CuSO_4 \cdot 5H_2O$ 10 g/L; $FeSO_4 \cdot 7H_2O$ 5.0 g/L) 7 mL; straw bleached pulp or wheat straw 20 g; distilled water up to 1000 mL; the medium pH has been set to 4.5. The culture duration: 120 h at a temperature of 30°C. After separating the micellium and the nondegraded material, there has been obtained the culture liquid whose enzyme activity has been determined against various substrates: filter paper (50 mg), solution of carboxymethyl cellulose (1 mL; 0.5%), or cellobiose (1 mL; 0.1%) by the method proposed by Mandels et al.[5] and modified by us by substituting the dinitrosalicylic acid with picric acid.[4]

Immobilization. The Ponilex type ion exchangers have been treated with an acetate buffer solution 0.02*M* (pH 4.5) and then contacted with an equivalent volume of culture liquid with known enzyme activity—under periodical stirring—followed by setting for 48 h at a temperature of 20°C. The enzyme preparations obtained by ionic or covalent bonding have been separated by supernatant by filtering and washing with acetate buffer, until the effluent showed no enzyme activity. In some cases, after immobilization, the enzyme preparations have been treated for 10 min with solutions of glutaraldehyde (1.25 or 2.5%) at ambient temperature. For all products thus obtained, the enzyme activity has been determined by the above-mentioned method,[4] using 1 g of wet preparation, 1 mL solution of acetate buffer, and 1 mL solution of cellobiose (0.1%) or CMC (0.5%).

Preparation of Alkaline Extracts. Alkaline extracts resulted from treatment of industrially obtained softwood or beech bark and of the *Asclepias syriaca* plant with solution of sodium hydroxide (1%), under previously mentioned conditions.[6,7] After acidification with acetic acid, up to a pH of 4.5 and separation by centrifugation of the material precipitated as a result of this treatment, the isolated liquid phases have been used in reactions of hydrolysis with the obtained enzyme preparations. The hemicellulose content, both in the initial samples and those obtained after hydrolysis, has been

determined by precipitation with ethyl alcohol, separation by centrifugation drying and weighing of the precipitates produced.

Enzymatic Hydrolysis. The enzyme preparations produced have been introduced in a column (L = 42 cm; ϕ = 2 cm) equipped with a jacket for reaching a temperature of 50°C. The CMC solutions (0.3%) in acetate buffer or those containing hemicellulose have been passed through the column, at a flow rate of 0.6 mL/min. Periodically, samples have been taken from the effluent collected, and by using the reaction with picric acid, the amount of reducing substances—expressed as glucose—has been determined. In experiments made with alkaline extracts from beech bark, hemicelluloses have been separated by precipitation with ethyl alcohol, dissolved again in acetate buffer solutions (0.02M, pH 4.5), and subjected to hydrolysis, under conditions previously mentioned. After being used in hydrolysis reactions, the enzyme preparations have been characterized from the viewpoint of their activity towards CMC and cellobiose solutions.

RESULTS AND DISCUSSION

The microorganisms considered are known to produce—depending on the substrate nature and on the culture conditions—a mixture of exocell enzymes involved in the degradation of the polysaccharide complex (hemicellulose and cellulose) or taking part in the metabolization of the reaction products or in the transformation of the lignin component (for example, oxidases).

Thus, in the case of the microorganisms *Aspergillus niger* and *Trichoderma viride*, in the culture media there could be identified, besides cellulase, hemicellulase enzymes (e.g., mannase, xylanase),[8,9] glucosidase,[10] and phenoloxidase[11]). These enzymes are characterized by ionic charge and different molecular dimensions. Consequently, one can appreciate that, during immobilization, the enzymes of the system may competitively interfere, the resulted preparations showing therefore a variable activity.

In order to immobilize the enzymes biosynthesized by the two microorganisms, different carriers have been used and, taking into account the conditions previously settled,[4] supplementary information upon the parameters influencing the immobilization process have been obtained (Table I).

From the determinations undertaken, one can observe a different affinity of the carriers used, as compared with the components of the enzyme complexes, which is determined—on the one hand—by the features of the ion exchanger and—on the other—by the nature of the enzymes present in the culture medium. Thus, in the case of the Ponilex AS-98 carrier, certain concentration effects of the β-glucosidase enzyme are to be observed, as compared with the culture liquids (samples 4, 10, and 16). The treatment of the enzyme preparations with glutaraldehyde, under determined conditions (samples 4 and 8) does not diminish the enzyme activity, being even advantageous. Interesting results may be obtained by immobilization of enzymes from mixtures of culture media (*T. viride* + *A. niger*), being known that their action is reciprocally completed during hydrolysis reactions.[6]

Experiments regarding the immobilization of cellulase enzymes on various supports have evidenced the fact that, for the estimation of the performances of the obtained products, activity determinations are not relevant, having to

TABLE I
Activity of the Enzyme Preparations Obtained on Ponilex-Type Carriers and of the Effluents Obtained during Immobilization

No.	Sample	Immobilization procedure	Enzyme activity (IU) Towards		
			Filter paper[b]	Carboxymethylcellulose[c]	Cellobiose[d]
1.	Broth liquid from *Aspergillus niger* (straw bleached sulphate pulp as substrate)	—	1.66	2.59	1.75
2.	Ponilex AS-98 carrier	Ionic	—	1.38	1.75
3.	Effluent	—	1.38	1.94	1.01
4.	Ponilex AS-98 carrier	Ionic, treatment with glutaraldehyde, 1.25%	—	1.11	3.33
5.	Ponilex AS-98 carrier	Ionic, treatment with glutaraldehyde, 2.5%	—	0.74	0.64
6.	Ponilex CC-31 carrier	Ionic	—	0.64	1.11
7.	Effluent	—	1.11	3.14	0.37

8.	Ponilex CC-31 carrier	Ionic, treatment with glutaraldehyde, 1.25%	—	1.29	1.20
9.	Broth liquid from *Aspergillus niger* (wheat straw as substrate)	—	1.39	2.50	1.50
10.	Ponilex AS-98 carrier	Ionic	—	0.42	3.04
11.	Effluent	—	0.64	0.97	2.28
12.	Broth liquid from *Trichoderma viride* (straw bleached pulp as substrate)		2.04	1.50	1.65
13.	Ponilex AS-60 carrier	Ionic	—	0.55	1.15
14.	Effluent	—	1.02	0.74	1.11
15.	Broth liquid mixture from *A. niger* + *T. viride* (2 :1) (wheat straw as substrate)	—	1.06	1.15	0.94
16.	Ponilex AS-98 carrier	covalent	—	1.89	2.31

[a]AS-98 = weak basic ion exchanger, exchange capacity, 0.31 meq/mL or 7.46 meq/g; AS-60 = weak basic ion exchanger, exchange capacity, 0.42 meq/mL or 6.74 meq/g; CC-31 = weak acid ion exchanger, exchange capacity, 1.44 meq/mL or 13.2 meq/g.

[b]Representing the activity of β-1,4 glucancellobiohydrolase (EC 3.2.1.91) and endo-β-1,4 glucanase (EC 3.2.1.4.).[12]

[c]Measuring the activity of β-1,4 glucanase, xylanase and mannanase.[13]

[d]Representing the activity of the β-1,4 glucosidase (EC 3.2.1.21).[12]

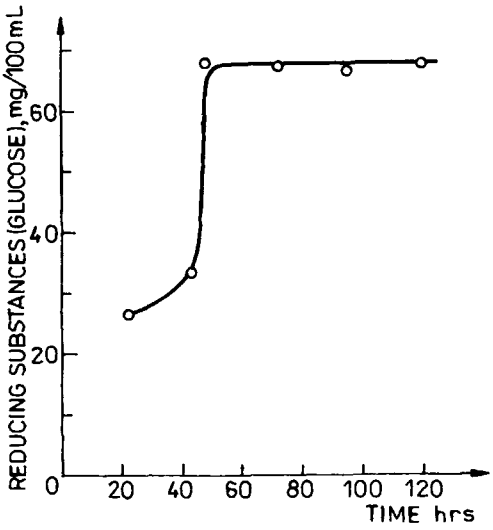


Fig. 1. Variation of the reducing substances (as glucose) content depending on the hydrolysis duration of the CMC solution (0.3%) in buffer acetate (0.02*M*, pH 4.5) with a preparation immobilized on Ponilex AS-98 (sample 4, Table I).

be completed by testing of the preparations in hydrolysis reactions. In this respect, hydrolysis reactions have been performed, by using CMC solutions (Fig. 1), hemicellulose from the alkaline extracts separated from *Asclepias syriaca* (Fig. 2) and wood bark, acidified with acetic acid, at a pH of 4.5, and polyoses, recovered by precipitation with ethyl alcohol from the alkaline

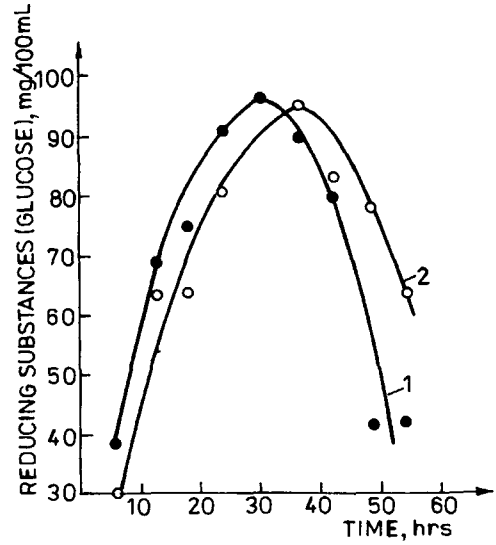


Fig. 2. Variation of the reducing substances (as glucose) content depending on the hydrolysis duration of the hemicellulose from alkaline extract of *Asclepias syriaca*, acidified with acetic acid (pH 4.5). Hydrolysis performed with sample 4 (1) and sample 16 (2).

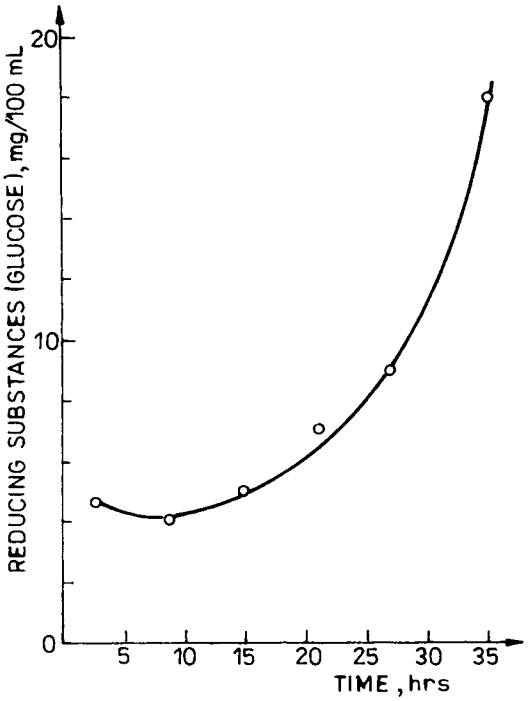


Fig. 3. Variation of the reducing substances (as glucose) content depending on the hydrolysis duration of the hemicellulose separated from the alkaline extract of beech bark, dissolved once more in buffer acetate solution (0.02*M*; pH 4.5). Hydrolysis performed with sample 4.

extract of beech bark (Fig. 3) and dissolved again in a solution of acetate buffer (0.02*M*; pH 4.5).

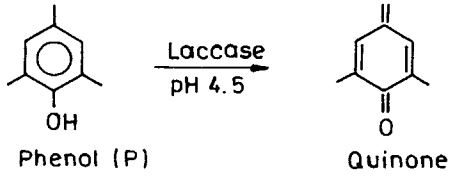
From the data recorded, one can observe that enzyme preparations act differently, depending on the substrate nature. Thus, in the case of the reaction of carboxymethylcellulose, the increase of the amount of reducing substances occurs up to a duration of 40 h, after which it remains constant. The situation could be explained by the accumulation of the substituted CMC units, which are no longer recognized by enzymes.

The hydrolysis of hemicellulose from the alkaline extract of *Asclepias syriaca*, acidified with acetic acid, at a pH of 4.5, occurs evidently with both enzymes preparations used, which is demonstrated by the continuous increase of the amount of released reducing substances, up to a time of 30–35 h. Afterwards, the decrease of sugar concentration may be observed, which could be caused by the presence of the phenol compounds as well as by the immobilization—on the carriers employed—of some phenoloxidase enzymes, besides the hemicellulase and cellulase ones. Such a phenomenon has been more evident in the hydrolysis performed with alkaline extract of beech bark when, in the resulted products, the reaction of coloring with picric acid characteristic to the reducing substances did not take place anymore, although the diminishing of the content of hemicellulose precipitable with alcohol, was observed (Table II). Recently, it has been noticed that the Ponilex AS-98 carrier manifests affinity not only for the enzymes that break

TABLE II
Behaviour of Some Enzyme Preparations in the Hydrolysis Reaction of Hemicellulose from Various Vegetal Alkaline Extracts.

Sample subjected to hydrolysis	Carrier used for the enzyme preparation	Time (h)	Hemicellulose (%)	Decrease of the hemicellulose content (%/h)
1. Alkaline extract from softwood bark	—	—	0.54	—
	CC-31	24	0.456	0.65
	AS-98	26	0.414	0.89
2. Alkaline extract from beech bark	—	—	0.34	—
	CC-31	7	0.167	7.27
	AS-98	10	0.17	3.5
Hemicellulose separated from the alkaline extract of beech bark	AS-98	35	0.09	2.1
3. Alkaline extract from *Asclepias syriaca*	—	—	0.50	—
	AS-98 (sample 4)	54	0.078	1.56
	AS-98 (sample 16)	54	0.12	1.40

polyoses, but also for the enzymes of the laccase type.[14] In such conditions, the activity of laccase which participate in an electron transport chain[15,16] is being stimulated by the presence of polyphenol compounds, the following reaction scheme becoming thus possible:



Phenol (P) Quinone (Q)

$$\text{Glucose} + Q + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic Acid} + P$$

The acids thus formed do not evidence color reactions with the picric acid any more.

Glucose oxidase is an enzyme characterizing the *A. niger* microorganism.[10] The formulated hypothesis has been confirmed during hydrolysis of the hemicellulose separated by precipitation with ethyl alcohol from the alkaline extract of beech bark. This time, the coloring of the reaction products with picric acid has been positive, spectrophotometrical following of the process being possible (Fig. 3). In the absence of phenol products, the amount of sugars released by hydrolysis increase continuously with the reaction time. At the same time, alkaline extracts from beech bark, acidified, in which there have been introduced 0.2% cellobiose, have been subjected to hydrolysis. In

such an experiment, no positive reaction has been observed for the interaction of the compounds resulted from hydrolysis with picric acid, although the hemicellulose content decreases from 0.34 to 0.23% after 39.5 h of reaction.

Data listed in Table II show that decrease of the hemicellulose content in various alkaline extracts depends on the polyose nature—conditioned by species as well as by that of the carrier used in the immobilization of the hydrolytic enzymes. The carrier, characterized by a certain retaining capacity, as against the components of the enzyme system, induce a certain ratio between them, which results in attaining a corresponding hydrolysis degree and reaction rate. On the other hand, the complex structure of hemicellulose, as well as the various types of linkages between structural monosaccharides, determine specific peculiarities in the hydrolysis reaction with the immobilized enzymes.

Preliminary trials regarding the influence of the ratio of some compounds (sample 16, Table I) by continuously increasing the contents of β-glucosidase, on using a culture media of *Aspergillus niger*, did not offer—for the time being—significant results, which is certainly due to the complexity of the system. At the same time, as already observed, the Ponilex AS-98 carrier is characterized by affinity towards laccase—which is an aspect that is still waiting to be thoroughly investigated. For the enzyme preparations used in hydrolysis reactions, determinations of the activity have evidenced its variable reductions, depending on the immobilization variant (ionic or covalent) chosen, on the nature of the substrate subjected to hydrolysis and also on the operating time. From this point of view, preliminary data obtained plead for the covalent immobilization, requiring at the same time the elucidation of the role played by some compounds accompanying polysaccharides (e.g., polyphenols) in the reaction of hydrolysis or in the modification of the activity of the enzyme preparations.

An additional investigation of the immobilization process will permit a special characterization of the preparation for other different substrate categories, too, besides those utilized up to now, with a view to obtaining more precise information upon the enzymes components retained from the complex. It is also important to find out an optimal ratio between the enzymes retained on the carriers considered, with a view to obtaining a maximum efficiency in their utilization.

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