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STUDIES ON THE PRODUCTION OF ENZYMIC PREPARATIONS FROM BIOTECHNOLOGICALLY IMPORTANT MICROMYCETES

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Abstract: Three species of micromycetes, *Fusarium oxysporum*, *Penicillium citrinum* and *Botrytis paeoniae*, were studied for production of the following enzymes: amylase, dextranase, levanase and inulinase. A mineral medium was used as a nutritive base, to which was added 2% or 0.2% of specific substrate (starch, dextran, levan and inulin). These served to induce the synthesis of the enzymes.

Enzymic activities were determined qualitatively by paper chromatography, and quantitatively by determining reducing sugars. It was established that most of the enzymes were secreted into the culture medium. Hydrolysis values of over 50% were recorded. A smaller quantity of each enzyme remained bound to the mycelium, and due to this, hydrolysis values were lower.

As regards the production of the five enzymes, the most active species was *Fusarium oxysporum*, then *Penicillium citrinum*. Several organic substances were used as growth stimulators and for the synthesis of the enzymes: yeast extract, maltose, sucrose and glucose. Yeast extract stimulated the production of all these enzymes in all three species; maltose stimulated the production of amylase, while glucose and sucrose stimulated the production of inulinase.

Keywords: amylase, dextranase, levanase, inulinase, micromycetes.

Introduction

Microorganisms represent a very important source of enzymes, and are used successfully in biotechnological applications for obtaining enzyme preparations. They present many advantages, by having a short or very short development period, and being easy to obtain in large quantities, on inexpensive media. In microorganisms the enzyme complement is complex and the concentration of various enzymes depends upon the species and is influenced by the culture medium.

Among microorganisms, microscopic fungi have a major use in biotechnology for realizing the useful manufacture of enzyme preparations [12].

Obtaining enzyme preparations from micromycetes has been the object of many studies. The N12 strain of *Aspergillus niger* has been used to obtain inulinase [13]. Inulinase is an exoenzyme (exoinulinase) which splits the β-2,1 type bonds, begining at the non-reducing end of inulin with release of fructose. Other strains of *Aspergillus niger* were used in the production of inulinase, as well as cellulase, xylanase, amylase and chitinase, some in large quantities. Both amylases and inulinases can be used in the food and textile industries [7]. Dextranases and levanases can be succesfully used in dental medicine [5].

Dextrans are polysaccharides of large molecular weight. There were identified bacteria and fungi that have enzyme complexes capable of hydrolyzing dextran [15].

Inulinase, which catalyses the hydrolysis of polyfructoses, is of special interest because fructose can be obtained from different vegetal sources. In the majority of microorganisms, the maximum biosynthesis of inulinase is at pH 3.5–8.5, the optimal temperature is 28–34 °C, but some strains produce the enzyme even at 40–50 °C [17].

In strains of *Rhizopus*, compounds that stimulate extracellular enzyme biosynthesis were tested. It was assessed that the stimulation effect of different compounds depends both on the hydrolytic enzymic compound and its concentration in the culture medium [3].

In strains of *Penicillium cyclopium, Cladosporium herbarum* and *Aspergillus niger*, the capacity was tested to produce metabolites capable of halting metal corrosion. The differences recorded in different fungal strains were significant so it is important to know the physiological characteristics of the strain in order to evaluate accurately the effectiveness of these fungi regarding metal corrosion [11].

Homogenous enzyme preparations were obtained from *Aspergillus versicolor* and were characterized molecularly. The cellulase complex includes three types of enzyme: endo-1,4-α-glucanase, exo-1,4-α-glucanase and α-glucanase. Endo-1,4-α-glucanase is important because it hydrolises the α-1,4 bond [8].

The use of filamentous fungi to produce enzyme preparations by different fermention processes is of special importance. Recently a set of enzymes were obtained by fermentation of different substrates [6, 14]: wheat bran (cellulases, xylanases and the polygalacturonase, pectinases, proteolytic enzymes), cellulose and starch (cellulase and amylase), lignocellulose (various enzymes), corncobs (cellulase and β-glucosidase), agricultural waste (cellulase, xylanase, β-glucosidase, carboxy-methyl-cellulase), beet pulp (polysaccharide enzymes, β-glucosidase), wheat straw (carboxy-methyl-cellulase, β-glucosidase, cellulase) and soya bran (pectinases).

Ordinarily the enzymes used in animal nutrition are hydrolases and are employed directly as food additives. Studies have been carried out involving the supplementation of endogenous digestive activity of animals with proteases and amylases [2, 9]. Likewise anti-nutritional factors such as β-glucans and phytic acids were removed from the food mass [16] and specific nutrients with increased availability in absorption were extracted to intensify the energetic value of the nutritive ingredients [10].

The aim of the present study was to obtain enzyme preparations of amylase, dextranase, levanase and inulinase from different micromycetes for their eventual biotechnological use.

Materials and Methods

The micromycetes studied were: *Fusarium oxysporum, Penicillium citrinum* and *Botrytis paeoniae*, strains available in the collection of the Microbiology Laboratory of Babeş-Bolyai University, Cluj-Napoca.

The culture media. The micromycete strains were grown on a basic mineral nutritive medium [1]. To this culture medium were added 2% or 0.2% of specific substrates such as: soluble starch (Reactivul preparation), dextran (Reanal preparation), inuline (Fluka preparation) and levan (preparation obtained in the Microbiology Lab). The culture media were distributed in Erlenmeyer flasks and sterilized at 121°C for 30 minutes. A few organic substances were used as stimulants for growth and synthesis of enzymes: yeast extract, maltose, sucrose and glucose. For inoculation young micromycete cultures grown on Czapek-Dox medium were used. Each micromycete was used to inoculate three media. Incubation was carried out at 28°C for 14 days. The mycelium was removed by filtration to obtain its biomass, which was dried at 40 °C for 48 hours. The culture liquid was centrifuged at 4000 rpm for 30 minutes. Reaction mixtures were prepared from the dried and ground biomass and also from the culture liquid for determining the activities of amylase, dextranase, levanase and inulinase.

The enzymes studied. The composition of the reaction mixtures was as follows:

- 9 ml culture liquid, 2 ml toluene (with aseptic function), 1 ml starch solution 20 %, 1 ml dextran solution 20 %, 1 ml levan solution 2.5 % or 1 ml inuline solution 2.5 %

- 200 mg mycelial biomass, 1.5 ml toluene (with aseptic function), 5 ml starch solution 2 %, 5 ml dextran solution 2 %, 5 ml levan solution 0.25 % or 5 ml inuline solution 0.25 %

Mixtures without enzymes were prepared as controls (without mycelial biomass or culture liquid, respectively) with just toluene and the enzyme substrates or with the mycelian biomass and culture liquid without the enzyme substrates (only toluene and distilled water), respectively. All the reaction mixtures were incubated for 24 hours at 37 °C.

Chromatographic analysis. After incubation the reaction mixtures were qualitatively analysed by chromatography on Whatman 1 paper and quantitatively analysed by dosing the reducing sugars via the Somogyi-Nelson method [4].

Results and Discussion

Enzyme activities were determined qualitatively by paper chromatography and quantitatively by determining reducing sugars. All the micromycetes studied displayed activity of the four enzymes amylase, dextranase, levanase and inulinase, with differences according to the species studied and type of enzyme activity (Table 1). Each micromycete species separately produced the four enzymes, the enzyme substrate constituting the specific indicator for each enzyme, also being the source of carbon and energy.

Table 1: Enzyme activities in the culture liquids studied

Micromycetes	Activity							
	amylase		dextranase		levanase		inulinase	
	mg glucose/ 10 ml	hydrolysis %	mg glucose/ 10 ml	hydrolysis %	mg glucose/ 10 ml	hydrolysis %	mg glucose /10 ml	hydrolysis %
Fusarium oxysporum	1.410	68.1	1.241	57.4	1.547	67.8	1.341	64.5
Penicillium citrinum	1.345	66.8	1.324	62.4	1.424	65.7	1.125	62.8
Botrytis paeoniae	1.184	60.2	0.987	49.8	1.292	58.1	0.983	50.2

Although each polysaccharidase was synthesized in the mycelium, the enzyme was chiefly released into the culture medium, acting hydrolytically on the substrate, resulting in hydrolysis products that the micromycete can assimilate, thus enabling the growth of mycelium and leading to the biosynthesis of new quantities of enzyme.

As seen in Fig. 1, the highest activity is that of amylase, which at all the species studied attained substrate hydrolysis values greater than 60 %. Next is levanase with hydrolysis values greater than 55 %. After amylase and levanase come inulinase generation with over 50 % and dextranase production, in which only in *Botrytis paeoniae* were hydrolysis values slightly below 50 %.

It can also be seen that the species with the greatest capacity to produce the enzymes studied is *Fusarium oxysporum*, followed by *Penicillium citrinum*, while lower values were recorded for *Botrytis paeoniae*. Thus, *Fusarium oxysporum* is the most widely organotrophic species, which can degrade each substrate in larger quantities.

Some of the enzymes studied stay bound to the mycelium, not being released into the culture medium. Quantitatively this element is smaller, yet it presents a special practical importance. It can be seen (Table 2) that the calculated hydrolysis percentages have smaller values, most of all for levanase and dextranase. It is also noticeable that hydrolysis intensity is more pronounced in the species *Fusarium oxysporum* and *Penicillium citrinum*.

Regarding the production of the four enzymes by the micromycete species studied it can be interpreted that *Fusarium oxysporum* presents the highest capacity for their synthesis, followed by *Penicillium citrinum* (Fig. 2). *Botrytis paeoniae* has a much less capacity for synthesis of these enzyme types and so is not a reliable source for biotechnological use.

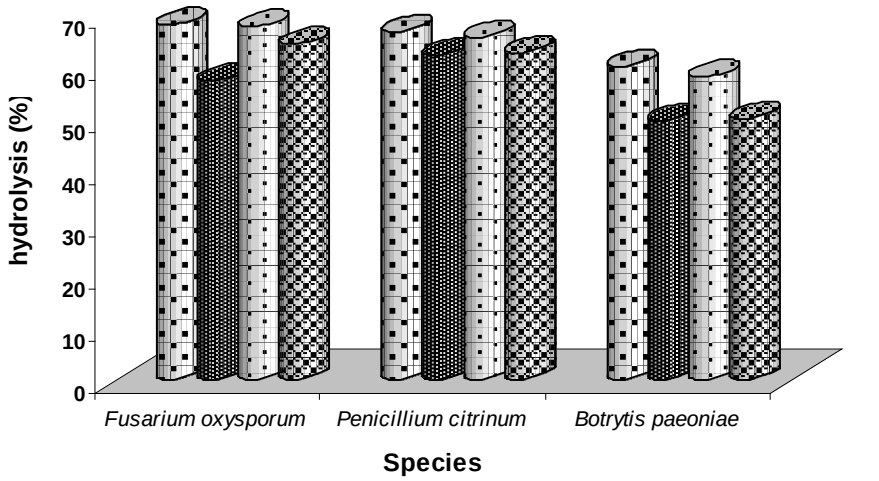

Fig. 1: Intensity of enzyme activities in the culture liquids

Table 2: Enzyme activity in the mycelium of the micromycetes studied

Micromycetes	Activity							
	amylase		dextranase		levanase		inulinase	
	mg glucose/ 10 ml	hydrolysis %	mg glucose/ 10 ml	hydrolysis %	mg glucose /10 ml	hydrolysis %	mg glucose/ 10 ml	hydrolysis %
Fusarium oxysporum	0.957	45	0.596	30	0.621	28	0.869	41
Penicillium citrinum	0.754	40	0.476	26	0.411	21	0.701	38
Botrytis paeoniae	0.652	29.5	0.382	19	0.286	17	0.692	31

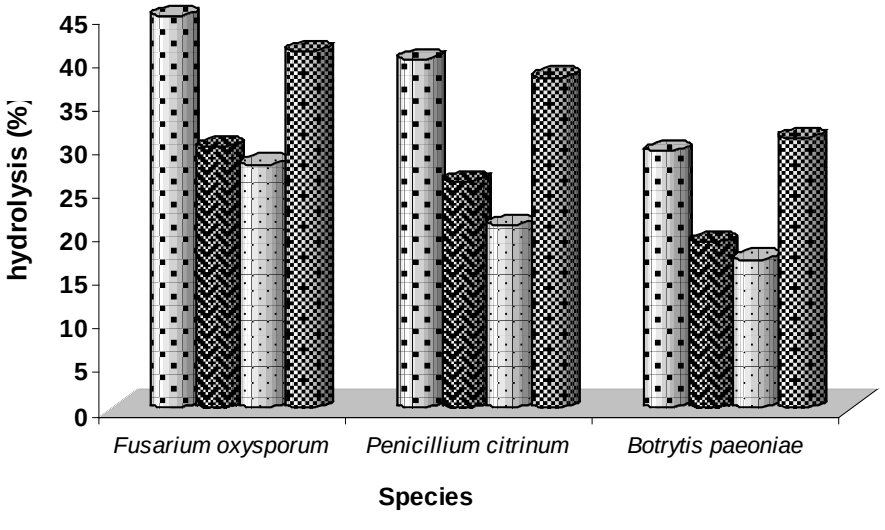

Fig. 2: Intensity of enzyme activities in the mycelium of the micromycetes

In order to increase the capacity for enzyme biosynthesis organic substances such as yeast extract, maltose, sucrose and glucose were added. The substances studied produced different effects according to microbial species.

In *Fusarium oxysporum*, yeast extract stimulated each enzyme activity, a fact attributed to its complex effect, which supplements the nitrogen source, while the purine and pyrimidine bases, the coenzymes it contains, represent genuine growth factors (Fig. 3). The presence of growth factors in the culture medium stimulates growth and biosynthetic activity. Inulinase synthesis was stimulated by yeast extract in *Arthrobacter* sp. [18]. Maltose only stimulated dextranase production, while sucrose and glucose did not induce any change in enzyme activity.

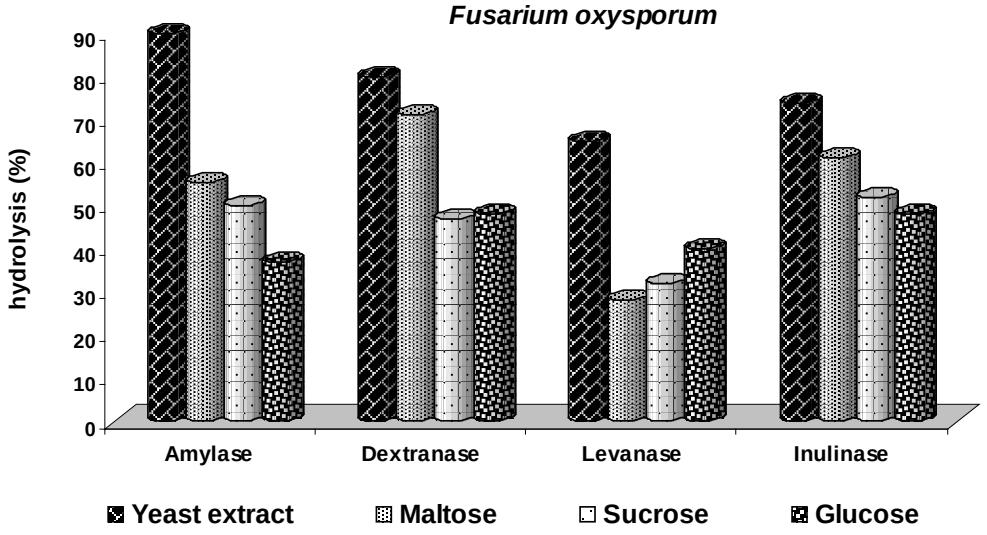

Fig. 3. Influence of growth stimulators on production of the enzymes studied

In *Penicillium citrinum* yeast extract stimulated the production of all the enzymes studied (Fig. 4). Maltose had a positive effect only on dextranase production. Added sucrose had no influence on the activities of the enzymes studied.

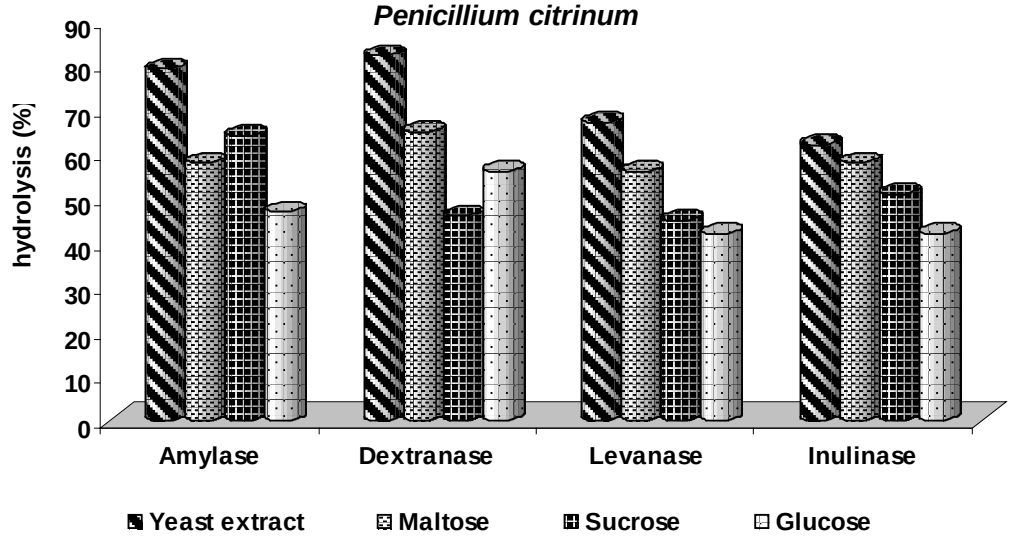

Fig. 4: Influence of growth stimulators on production of the enzymes

In *Botrytis paeoniae* yeast extract stimulated the activity of all the enzymes. Maltose had a positive effect on amylase and inulinase activity while sucrose stimulated amylase and inulinase production (Fig. 5).

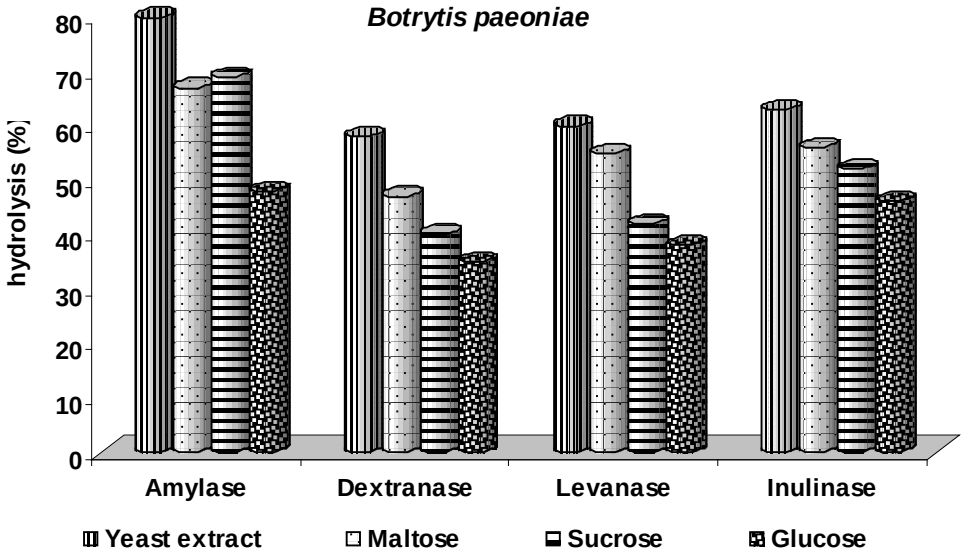

Fig. 5: Influence of growth stimulators on the production of the enzymes

As regards the production of the four enzymes by the agency of growth stimulators added to the culture medium, the most active species was *Fusarium oxysporum*, followed by *Penicillium citrinum.* Among the stimulators, yeast extract stimulated the production of all these enzymes in all the micromycete species. The data obtained indicate that some of the tested organic substances stimulated the activity of one or more enzymes but none of them caused inhibition.

Conclusions

The micromycetes studied (*Fusarium oxysporum, Penicillium citrinum, Botrytis paeoniae)* produce polysaccharidases such as amylase, dextranase, levanase and inulinase, which can be used in biotechnology for obtaining enzyme preparations.

Of the micromycetes studied, the most active in polysaccharidase production in the culture medium is *Fusarium oxysporum*, especially with regard to amylase and levanase in which hydrolysis was calculated as greater than 64 %.

All the polysaccharidases studied proved to be inductible, adaptative enzymes, the specific inductor being the enzyme substrate, which led to obtaining greater quantities of enzyme.

The organic substances added to the culture medium stimulated polysaccharidase synthesis according to the substance type and the microbial species which produced the enzyme. The most efficient added substance was yeast extract, which intensified all the enzyme activities.

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STUDII PRIVIND OBȚINEREA UNOR PREPARATE ENZIMATICE DIN MICROMICETE CU IMPORTANȚĂ BIOTEHNOLOGICĂ

(Rezumat)

Au fost studiate trei specii de micromicete: *Fusarium oxysporum, Penicillium citrinum, Botrytis paeoniae,* pentru a produce următoarele enzime: amilază, dextranază, levanază şi inulinază. Ca şi mediu nutritiv s-a folosit un mediu mineral la care s-a adăugat 2% sau 0,2% din substraturile specifice (amidon, dextran, levan şi inulină). Acestea trebuiau să inducă producerea de enzime specifice.

Activitățile enzimatice au fost determinate din punct de vedere calitativ prin cromatografie pe hârtie, iar din punct de vedere cantitativ prin determinarea zaharurilor reducătoare. S-a stabilit că cea mai mare cantitate din fiecare enzimă a fost secretată în mediul de cultură.

S-au înregistrat valori ale hidrolizei de peste 50%. O cantitate mai mică din fiecare enzimă a rămas captată în miceliu iar din această cauză valorile hidrolizei au fost de doar 10-40%.

În ceea ce privește producerea celor 4 enzime cea mai activă a fost *Fusarium oxysporum*, urmată de *Penicillium citrinum*. Câteva substanțe organice au fost folosite ca şi stimulatori pentru creşterea şi sinteza enzimelor: extract de drojdie, maltoză, zaharoză şi glucoză. Extractul de drojdie a stimulat producerea tuturor enzimelor menționate la toate speciile, maltoza a stimulat producerea de amilază iar glucoza şi zaharoza au stimulat producerea de inulază.

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