INFLUENCE OF SOME EXOGENOUS FACTORS ON γ AMYLASE ACTIVITY IN FUNGUS *RHIZOPUS STOLONIFER*

INFLUENȚA UNOR FACTORI EXOGENI ASUPRA ACTIVITĂȚII γ AMILAZEI LA CIUPERCA *RHIZOPUS STOLONIFER*

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Abstract. *Microbial amylases are present among bacteria, protozoa, algae and fungi, and fungal amylases are found especially in species of Aspergillus and Rhizopus. The use of these microorganisms in industrial scale production has obvious advantages, including rapid multiplication rate, diversity of present enzymes and genetic manipulation. Synthetic media are costly and therefore the use of cheap and available raw materials such as grain caryopses with a high starch content is required. The objective of this paper is to monitor the influence of nutritive substrate represented by grinded cereal caryopses, the pH, trace elements and amino acids on the γ amylase activity from fungus Rhizopus stolonifer. Enzymatic measurements were performed at three time intervals: 3, 6 and 9 days. A stimulatory action exerted amino acids: histidine, methionine, glutamic acid and trace elements: manganese, copper and trace elements mixture, and the optimum pH of the enzyme activity was 7 for media with corn, 6 for media with wheat and 4 for media with barley.*
Key words: *Rhizopus stolonifer, γ amylase, pH, amino acids, trace elements, cereal caryopses.*

Rezumat*. Amilazele microbiene sunt prezente la bacterii, protozoare, alge și ciuperci, iar cele fungice se găsesc mai ales la speciile de Aspergillus și Rhizopus. Utilizarea acestor microorganisme în producția la scară industrială are avantaje evidente, ce includ rata rapidă de multiplicare, diversitatea enzimelor prezente și posibilitatea de manipulare genetică. Mediile de cultură sintetice sunt costisitore de aceea se impune folosirea unor materii prime ieftine și ușor accesibile cum sunt cariopsele de cereale cu un conținut ridicat de amidon. Obiectivul acestei lucrări reprezintă monitorizarea influenței substratului nutritiv reprezentat de cariopse măcinate de cereale, al pH-lui și al aminoacizilor asupra activității γ amilazei la ciuperca Rhizopus stolonifer. Măsurătorile enzimatice au fost efectuate la 3 intervale de timp: 3, 6 și 9 zile. O acțiune stimulatoare au exercitat aminoacizii: histidina, metionina, acidul glutamic și oligoelementele: manganul, cuprul și mixtura de oligoelemente, iar pH-ul optim de acțiune al enzimei a fost 7 în cazul mediilor cu porumb, 6 în cazul celor cu grâu și 4 în cazul mediilor cu orz.*
Cuvinte cheie*: Rhizopus stolonifer, γ amilaza, pH, aminoacizi, oligoelemente, cariopse măcinate*

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INTRODUCTION

Microbial amylases are present among bacteria, protozoa, algae and fungi, and fungal amylases are found mainly in species of *Aspergillus* and *Rhizopus* (Fisher and Stein, 1960). The use of these microorganisms in production an industrial scale has obvious advantages, including the rapid rate of multiplication, the diversity of present enzymes and possibility of genetic manipulation. Usually fungal amylase production was achieved with well defined chemical culture media by submerged fermentation or solid state fermentation (Ayogu and Amadi, 2010). However, these culture media are costly and therefore it requires the use of cheap and readily available row materials such as cereal caryopses with high starch content, which makes them excellent growth nutritive substrates for amylase producing microorganisms.

The glucoamylase is a hydrolyzing enzyme that can degrade both the amylose and amylopectin true hydrolyzing α-1,4 and α-1,6 glycosidic bonds of starch to produce glucose. Since can completely convert starch to glucose, glucoamyalase is currently one of the most important enzyme in the food industry (Ono et al., 1964; Beauchat 1987; Soccol et al., 1992), being used for the production of glucose and fructose syrup from liquefied starch. It also is used in baking and many food industries based on fermentation for commercial production and in some cases in skin and detergents industries (Hesseltine, 1965; Reed and Rhim, 1987; Whistler et al., 1984). This enzyme is produced by a variety of fungal species (Tsujisaka et al., 1958; Sen and Chakarabarty, 1984), although commercial supplies are primarily obtained from *Aspergillus* and *Rhizopus* sp. as a result of their low trans glycosylation activity and the ability to obtain near 100% yields of glucose from starch (Mertens and Skory, 2007). Due to its increasing demand, the production technique of glucoamylase has been studied in detail (Nahar et al., 2008).

This study aimed to investigate the influence of nutritive substrate represented by grinded cereal caryopses, the pH, trace elements and amino acids on the activity of γ amylase from *Rhizopus stolonifer* grown on liquid medium.

MATERIAL AND METHOD

Studied species – *Rhizopus stolonifer* was isolated from germinated cereal caryopses. In order to monitor the effect of pH on γ amylase activity was used liquid Leonian medium ($K_2HPO4.7H_2O$ 1.25 g, $MgSO_4$ 0.625 g, peptone 1 g, glucose 20 g, distilled water 1000ml) (Constantinescu O., 1974), from whose composition carbon source – glucose was substituted with wheat, corn and barley caryopses (20 g/l). The medium was distributed in 100 ml quantities in Erlenmeyer flasks. The flasks inoculated with *Rhizopus stolonifer* were incubated at 28° C. We have obtained 7 working variants for each type of cereal caryopses with a pH scale ranging between 2 and 8, the medium pH being adjusted using NaOH and H_3PO_4 solutions.

To follow the influence of some amino acids on the activity of glucoamylase from saprophytic fungus *Rhizopus stolonifer* the nitrogen source represented by peptone was replaced with the following amino acids: α alanine, valine, methionine, glutamic acid, serine, histidine, asparagine and a control variant deprived of any nitrogen source.

In order to determine the influence of some trace elements the medium was supplemented with the following trace elements: B – 10 mg, Cu – 100 mg, Mn – 20 mg, Mo – 20 mg, Fe – 20 mg, Zn – 200 mg, Pb – 20 mg. These quantities were calculated from the following compounds: H_3BO_3, $CuSO_4$ x $5H_2O$, $MnCl_2$ x $4H_2O$, $NaMoO_4$ x $2H_2O$, $FeSO_4$ x $7H_2O$, $ZnSO_4$ x $7H_2O$, $Pb(CH_3COO)_2$ x $3H_2O$. The control was deprived of trace elements. Finally we obtained 8 medium variants for each cereal species, and also a variant supplemented with a solution of trace elements listed above.

Enzyme assays and determination of soluble proteins amount were performed at 3 time intervals from fungus inoculation: at 3, 6 and 9 days, being carried out from fungus culture liquid. The γ amylase measurement was performed using dinitrosalicylic reagent method (Cojocaru, 2009). The enzyme activity was reported to the amount of total protein estimated by Bradford method (Artenie et al., 2008). Experiments were performed in triplicate for the accuracy of obtained data.

RESULTS AND DISCUSSIONS

Optimum pH range of glucoamylase varies between 4.5 and 5, with stability at pH 7 (Taylor P.M., et al., 1978), and according to James J.A. and Lee B.H. (1997) the optimum pH of glucoamylase varies between 3.7 and 7.4. *Rhizopus oligosporus* and *Rhizopus*-RFF strain showed maximum γ amylase activity when they were grown at initial pH 4.5 (Jin B., et al., 1999; Nahar S., et al., 2008).

The influence of pH on glucoamylase activity determined at three time intervals from *Rhizopus stolonifer* culture liquid is depicted in figure 1. Thus, in the first time period (3 days) from fungus inoculation enzyme activity is maintained at low levels in all medium variants, regardless of nutritive substrate used. Maximum values of enzyme activity were obtained at pH=5 for media with wheat (16.259 U/ml/mg protein), at pH 7 for those with corn (36.331 U/ml/mg protein) and at pH 8 for those with barley (23.991 U/ml/mg protein). In the next time interval a significant increase in enzyme activity takes place in most experimental variants. The highest enzyme activity has been recorded at pH 6 for media with wheat (19.945 U/ml/mg protein), at pH 7 for those with corn (48.348 U/ml/mg protein) and at pH 4 for those with barley (38.412 U/ml/mg protein). In the last interval of the experiment a sharp decrease occurs in the γ amylase activity in all work variants. The highest values were observed at pH 8 for media with wheat (9.547 U/ml/mg protein), at pH 7 for those with corn (16.139 U/ml/mg protein) and at pH 4 for those with barley (17.158 U/ml/mg protein).

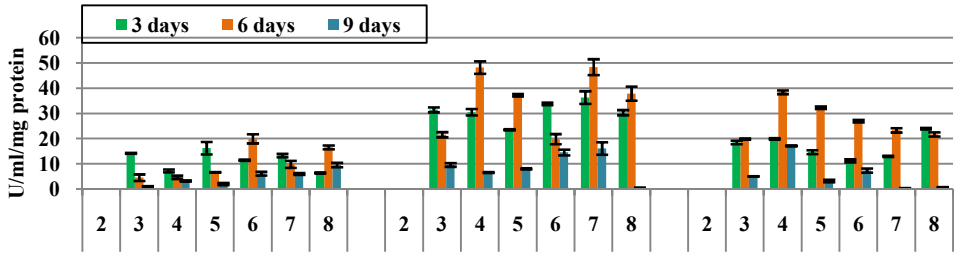

Fig. 1 - Influence of pH on γ amylase activity in fungus *Rhizopus stolonifer*

The influence of nitrogen source represented by a series of organic acids on γ amylase activity is shown in figure 2. After three days of incubation the enzyme activity was stimulated by the presence in the culture medium of glutamic acid (7.967 U/ml/mg protein), asparagine (10.628 U/ml/mg protein) and histidine (9.531 U/ml/mg protein) in media containing wheat caryopses, by the presence of histidine (15.095 U/ml/mg protein) and valine (18.623 U/ml/mg protein) in media with corn and by the presence of histidine (23.831 U/ml/mg protein) in the case of media with barley caryopses. At 6 days after fungus inoculation γ amylase was positively affected by all amino acids used in medium variants with wheat, the highest values being observed in variants with histidine (18.409 U/ml/mg protein) and methionine (15.78 U/ml/mg protein). α Alanine (9.489 U/ml/mg protein), asparagine (11.012 U/ml/mg protein) and valine (8.688 U/ml/mg protein) have a positive effect on the enzymatic activity in media with corn and regarding media with barley enzyme activity was stimulated by all amino acids, and the highest values were observed in the variants with α alanine (10.051 U/ml/mg protein) and methionine (9.489 U/ml/mg protein). In the last time interval glucoamylase activity was enhanced by the presence of all amino acids in media with wheat and barley, except for variant with valine (11.79 U/ml/mg protein) from wheat media and variant with methionine (4.442 U/ml/mg protein) from barley media. Also, the presence of asparagine (19.83 U/ml/mg protein) stimulated enzyme activity in the case of corn media. The highest values of γ amylase activity were observed in variants whit histidine (29.351 U/ml/mg protein) and methionine (31.874 U/ml/mg protein) for medium variants with wheat and in variants with histidine (14.87 U/ml/mg protein) and serine (18.543 U/ml/mg protein) for media with barley.

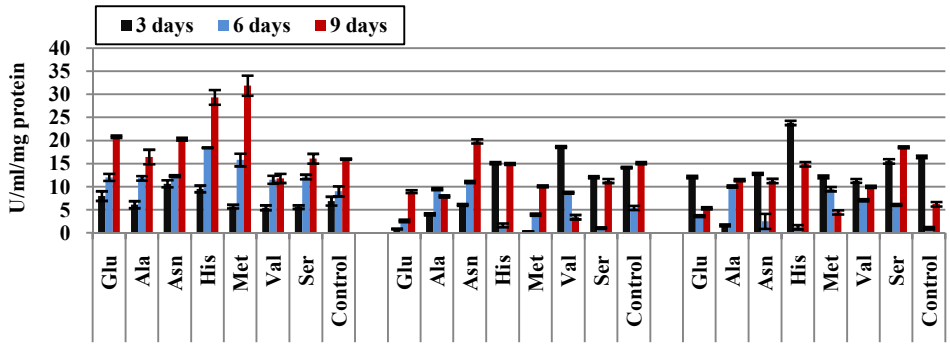

Fig.2 - Influence of amino acids on γ amylase activity in fungus *Rhizopus stolonifer*

To observe the influence of mineral elements on the glucoamylase activity, salts of these elements were added to the culture medium of fungus *Rhizopus stolonifer*. γ Amylase activity (figure 3) recorded, after 3 days of incubation, very high values in most work variants with grinded cereal caryopses. In medium variants with wheat caryopses a positive effect on the enzyme activity have had copper (30.144 U/ml/mg protein), manganese (57.482 U/ml/mg protein),

molybdenum (28.991 U/ml/mg protein), lead (26.491 U/ml/mg protein) and trace elements solution (66.491 U/ml/mg protein) and regarding medium variants with grinded corn caryopses most trace elements stimulated enzyme activity, except for the variant with iron (0,4292 U/ml/mg protein). Boron (9.811 U/ml/mg protein), copper (4.842 U/ml/mg protein), molybdenum (11.491 U/ml/mg protein), iron (13.575 U/ml/mg protein), lead (17.742 U/ml/mg protein) and the mixture of trace elements (13.736 U/ml/mg protein) have increased glucoamylase activity in medium set with barley. In the next time period there is a decrease in γ amylase activity in most work variants. In culture media that contained wheat amylase activity was stimulated by the presence of copper (28.027 U/ml/mg protein) and the trace elements mixture (22.161 U/ml/mg protein), while in the media supplemented with corn only molybdenum (22.067 U/ml/mg protein) stimulated enzyme activity. In the medium samples with barley enzyme activity was stimulated by manganese (17.582 U/ml/mg protein), zinc (15.495 U/ml/mg protein) and lead (23.187 U/ml/mg protein). In the last time interval γ amylase activity is increasing in most experimental variants. The enzyme activity was stimulated by the presence in the culture liquid of boron (33.798 U/ml/mg protein), copper (32.644 U/ml/mg protein), molybdenum (32.644 U/ml/mg protein), lead (40.047 U/ml/mg protein) in media with wheat; of all trace elements, except iron (10.532 U/ml/mg protein) regarding those with corn; of molybdenum (12.934 U/ml/mg protein) and the trace elements mixture (38.704 U/ml/mg protein) in the case of media with barley.

Manganese has a stimulating action on the glucoamylase activity synthesized by microorganisms (Pandey A., et al., 2000), as in the case of liquid cultures of *Rhizopus stolonifer*, medium variants supplemented with manganese showed quite high values.

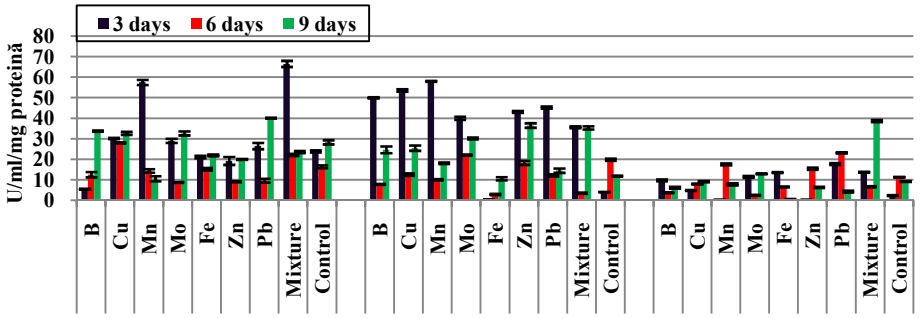

Fig. 3 -Influence of trace elements on γ amylase activity in fungus *Rhizopus stolonifer*

CONCLUSIONS

The maximum value of glucoamylase activity was recorded after 6 days of incubation at pH 6 for media with wheat, at pH 7 after for those with corn and at pH 4 for medium variants with barley caryopses. At the extreme value of pH 2 the fungus mycelium was completely inhibited during entire experiment. A number of amino acids have increased amylase activity: asparagines, histidine,

methionine, valine. Also, some trace elements that supplemented *Rhizopus stolonifer* culture medium had a positive effect on enzyme activity: boron, copper, manganese, zinc and trace elements solution.

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