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BIOSYNTHESIS AND PROPERTIES OF beta-1,3-GLUCANASES OF *Trichoderma hamatum*

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ABSTRACT

The strain of *Trichoderma hamatum* C-1 is able to produce extracellular lytic enzymes i.e. ß-1,3- glucanases, chitinases, ß-1,6-glucanases in the shaked cultures in the base medium enriched with fodder yeast supplemented with Avicel cellulose, as well as with glucose. Glucose had a repressive effect on ß-1,3- glucanase activity only when the fungus was cultivated in a high quantity (3%) of glucose medium. The enzymes were precipitated from liquid culture medium by ethanol and then optimal conditions for the enzymatic activity (pH 3.0-5.0; 50°C) and stability (pH 5.0; 50°C) of ß-1,3-glucanases were determined. The enzyme activity was strongly inhibited in the presence of $HgCl_2$, EDTA and stimulated by cations such as Mg^{+2} and Ca^{+2}. After enzymatic hydrolysis (120 min.)of laminarin products of reaction were measured using HPLC and about 90% of glucose and a little laminaribiose and laminaritriose were obtained. The enzymatic preparation was used for Yarrowia lipolytica A.101.1.31 and Saccharomyces cerevisiae protoplast production (100% after 4h and 100% after 2h respectively).

Key words: lytic anzymes, beta-1,3-glucanases, biosynthesis, biochemical properties, *Trichoderma hamatum*

INTRODUCTION

The extracellular complex of ß-glucanases produced by fungi of *Trichoderma hamatum* is able to hydrolyse ß-1,3, ß-1,4 and ß-1,6 bonds of ß-glucanes of plant or microorganism cell wall biopolymers. The ß-glucanases from *Trichoderma spp.* are important enzymes used in genetic manipulation (DNA separation, protoplast formation) and in releasing protein and pigments from cells. The ability of ß-1,3-glucanases to degrade the yeast cell wall seems to indicate that this enzyme may be helpful in the future as a factor increasing the digestibility of protein feed of microbiological origin. In recent years the cell wall degrading enzymes have often been used for biological control of soilborne pathogenic fungi.

MATERIALS AND METHODS

Microorganism and culture conditions. The strain *Trichoderma hamatum C-1* from our own collection was used in these studies. The strain was stored on potato-dextrose slants at 4°C. Duplicate shaking cultures were carried out on a mineral Saunders medium (Witkowska, Stempniewicz, Maj, 1999) enriched with yeast extract (0.1%) and wheat germs (0.1%). As carbon source, addition of fodder yeast (with or without Avicel cellulose) or glucose was used. The medium was inoculated with conidial suspension of $1x10^7$ cells in 1ml of 1% Tween 80. The cultures were conducted in 500ml flasks (100ml of medium) at 28°C for 7days. Samples for enzymatic determination were collected after centrifugation every 24h.

The enzymatic preparation (powder) was obtained after ethanol (4vol.) precipitation of enzymatic protein from postcultural liquid.

Enzyme assays Activities of beta-1,3-glucanases, beta-1,6-glucanases and beta-1,3(1,4) glucanases (Santos et al., 1977) were determined using laminaryn (6.5g/L), (Sigma) or pustulan (5g/L), (Sigma) or lichenan (5.0g/L), (Sigma), respectively as a substrate in 0.05M/L acetic buffer, at 50°, pH4.8. Activities of cellulases (Mandels et al.) were determined using NaCMC (10g/L) as a substrate in 0.05M acetic buffer, pH 4.8, at 50°C. Activities of chitinases (Witkowska et al., 1999) were determined using colloidal chitin (6.0g/L), (Sigma) as a substrate in0.05M acetic buffer at 40°C. The reducing substances – products of enzymatic reaction, were determined colometrically by the Miller`s method (DNS) using dinitrosalicylic acid (Sigma), The activities of examined enzymes were expressed in μmol of reducing substances/ml/min. (U/ml or U/mg of protein).The Lowry method was used to determine the protein content.

The effect of pH on the activity of beta-1,3-glucanases: solutions of 0.2% of enzyme preparation in 0.05M acetic buffer of various pH (3.0; 4.0; 5.0; 6.0; 7.0; 8.0) were used (50°C).

The effect of pH on the stability of beta-1,3-glucanases: solutions of 0.2% of enzyme preparation of pH 3.0; 4.0; 5.0; 6.0; 7.0; 8.0 were preincubated for 24h in 4°C, then activities of enzymes were determined at pH 4.8, 50°C.
The effect of temperature on the activity of beta-1,3-glucanases: solutions of 0.2% of enzyme preparation in 0.05M acetic buffer at various temperatures (40; 50; 60; 70; 80°C) were used.

beta-1,3-glucanases thermostabilty: solutions of 0.2%of enzyme preparation in 0.05M acetic buffer were preincubated at various temperatures (40; 50; 60; 70; 80°C) for 60min. and then activities of enzymes were determined at pH 4.8, 50°C.

The effect of activator and inhibitor solutions of 0.2% of enzyme preparation in 0.05M acetic buffer were preincubated for 15min. in 50°C in the presence of 2mM of each of the following substances: EDTA, $HgCl_2$, $CaCl_2$, $ZnSO_4$, $CuSO_4$, $MgSO_4$ and the activities of enzymes were determined by the standard assay procedure.
The laminarine (1%) hydrolysis process: was carried at 50°C. pH 4.8 for 30 and 120min. Reducing substances and glucose as final products of hydrolysis were determined with DNS and enzymatic methods (with glucoxidase and peroxidase), respectively. HPLC was used to determine the products of hydrolysis, too (Aminex HPX 87H column; 0.01M H_2SO_4 as eluent; flow - 0.3ml/min, detection refractometrical RF).

In production of yeast cell protoplasts, the cells of *Yarrowia lipolytica A101.1.31.* and *Saccharomyces cerevisiae* from 14h of culture (old) in YM medium were used. After cultivation, the culture was centrifuged (3500xg), the cells were washed twice with distilled water, suspended in a pre-treatment solution (1% ß-mercaptoethanol and 1% EDTA), (Sakai et al., 1986) and incubated at 28°C for 30min. Yeast suspension was standardised at 10^8 cell/ml (Campbell and Duffus, 1988). After incubation, the cells were centrifuged, washed twice with 0.6M/L KCl and suspended in lytic medium (60mM/L phosphate buffer pH7.5 and 10mM/L of ß-mercaptoethanol), (Sakai et al.,1986). The lytic medium contained enzymatic preparation from *Trichoderma hamatum* or commercial preparation (Sigma), (150mg/ml). The samples were incubated at 28°C. The degree of protoplast production was controlled using microscopy.

RESULTS AND DISCUSSION

The selection of a suitable carbon and energy source has a particular importance in the process of extracellular production of hydrolases by filamentous fungi. Very often this carbon and energy source plays the role of an inductor of enzymes. Many authors, in their works, stress induction feature of extracellular hydrolases of *Trichoderma* fungi (beta-1,3-glucanases, cellulases, chitinases) and positive influence of the added stimulators present in waste fungi mycelium, in yeasts biomass , and in cell walls of fungi and yeasts, etc. on those enzymes

[15,17,20]. In this work, the addition of fodder yeast (D) specially in 4% concentration (together with 2% of AC) to the cultures of *T. hamatum* C-1 was the most suitable for biosynthesis process of beta-1,3-glucanase(12U/ml), (Fig.1). Biopolymers of cell walls present in yeast biomass have a stimulating influence on synthesis of those enzymes (Fig.1). The simultaneous addition of yeast and Avicel cellulose (AC) has a stimulating influence especially in case of addition of 4% of yeasts and of 2% of AC (Fig.1). That effect was not observed in the other combinations of inductors which were tested, and the maximal beta-1,3-glucanase activities did not excess 10U/ml. Rudawska & all.[10] in their works on induced biosynthesis of beta-1,3-glucanase, have obtained the activity not exceeding 0,9U/ml. Sandahu [12] obtained the beta-1,3-glucanases with activity of 0.28U/ml in the culture of *T.harzianum* with addition of mycelium of *Trichoderma* sp., and Targoński & Wójcik [16] obtained those enzymes with activity of 2.17 U/ml in case of *T.reesei* M.-7 growing in the presence of cellulose. In the authors' own studies it was proved that *T. hamatum* C-1 was able to produce beta-1,3-glucanases, not repressed by the presence of glucose in the medium (fig.2). The highest values of activities 7.7 U/ml were obtained in the presence of 1.5 % glucose after 7 days of culture, however an increased amount of glucose in the medium, in the range of 2% and 3% inhibited the biosynthesis of the studied enzymes, in~50% and~70 %, respectively.

Fig 1. The effect of addition of fodder yeast (FY) with and without Avicel cellulose (AC) on beta-1,3-glucanase biosynthesis in *T.hamatum C-1* culture.

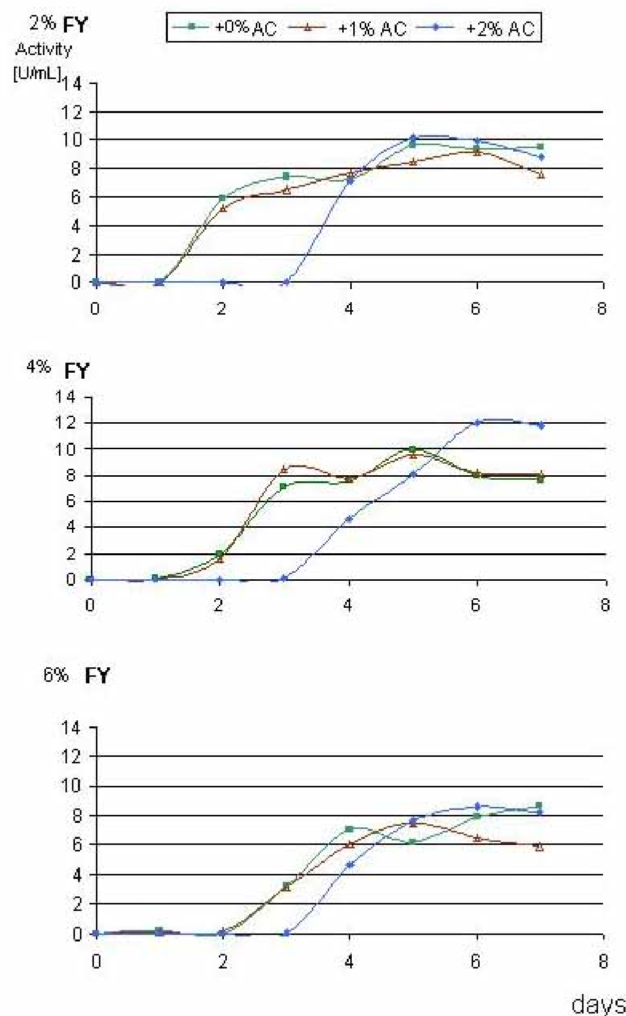
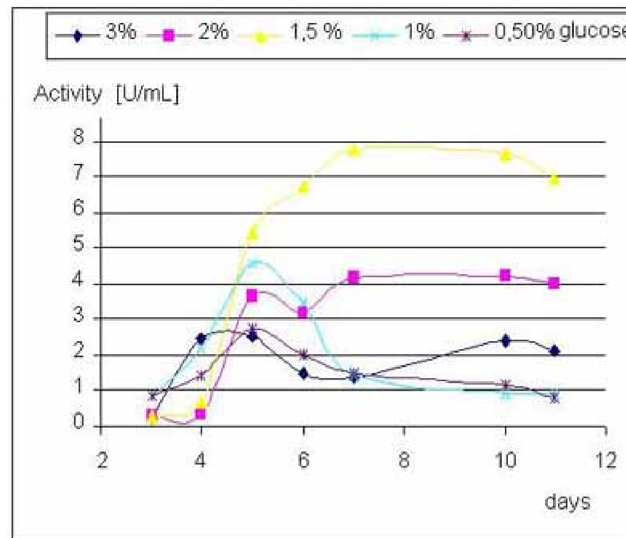


Fig 2. The effect of glucose on beta-1,3-glucanase biosynthesis in *T. hamatum C-1* culture..



Further studies on the properties of beta-1,3-glucanases were made on an enzymatic preparation obtained from the culture medium by ethanol precipitation of proteins. Some biochemical properties of beta-1,3-glucanases present in so obtained preparation were studied, such as pH and temperature range activity, and the action of some inhibitors or inductors on the activity and enzyme stability. Moreover, activities of other associated enzymes were measured. In those enzymatic preparations, besides the activity of beta-1,3-glucanases (1034U/g), the activity of cellulases (38.3 U/g) , beta-1,6-glucanases (9.0U/g) and beta-1,3(1,4)-glucanases (1482U/g) was present (Table 1). Beta-1,3-glucanases, present in enzymatic preparation were characterised by the highest activity at pH of 3.0-5.0, at the temperature of 50°C, and were stable at pH of 5.0 and temperature of 50°C (Fig. 3.). In many publications, the difference between the optimal values of pH and temperature of activity of beta-1,3-glucanases from different sources were reported. In Kitamoto [3] studies on properties of egzo-beta-1,3-glucanases from *T. harzianum* the optimal activity of those enzymes was at pH of 4.6 and 45°C. Maximal activity values of beta-1,3-glucanases from *T.viride*, Merc & Galas [7] obtained at pH 6.5 and temperature of 45°C. Norhona & Ulhoa [9] obtained beta-1,3-glucanases from *T. harzianum*, at pH 4.4 and temperature of 45°C, and Sharma & Nakas [14] observed the highest activity of beta-1,3-glucanases from *T.longibrachiatum* at pH 3.0-5.0 and temperature range of 30-70°C. The biochemical properties, i.e. inhibitors and activators are also important in the characteristic of enzyme, because they make it possible to establish such conditions of application that do not decrease the action of lytic enzymes. In the present work beta-1,3-glucanases from *T.hamatum* have shown about 20% increased activity in the presence of Ca^{+2} and Mg^{+2}, an insignificantly increased activity in the presence of Cu^{+2} and Zn^{+2}, and were inhibited by $HgCl_2$ and EDTA (Fig.4). That results implied the presence of SH containing amino acid residues in the active site and the role of metal ions in enzymatic catalysis. Totsuka & Usui [18] in the study on enzymes from *Rhizoctonia solani* evaluated the influence of metal ions on activity of beta-1,3-glucanases and an important inactivation by Hg^{+2} (decrease of activity by 87%), and a small effect of Mg^{+2},Ca^{+2}, Zn^{+2}, Fe^{+2}, Pb^{+2} and Cu^{+2} were observed.

In the present work, characteristics of hydrolytic process of laminaryn as the substrate by beta-1,3-glucanases from *T.hamatum* C-1 was studied. Degradation products were analysed by HPLC and colorimetric measure (glucose was measured enzymatically). After 30 min of hydrolysis, about 30 % of glucose was detected and a small amount of laminarytriose, and after 120 min of hydrolysis near 90% of glucose and a small amount of laminarybiose and laminarytriose (Fig. 5. Table 2). Those results have shown the synergetic action of egzo and endo glucanases that conducted the process to glucose. De la Cruz [1] detected in enzymatic hydrolyses mainly the presence of glucose, laminarybiose and laminaryterose after the hydrolysis process of laminaryn by enzymes from *T.harzianum* CECT 2413 culture.

Table 1. Characteristics of *T.hamatum C-1* enzyme preparation.

ACTIVITY (U/g of preparation)				
Beta-1,3-glucanase	Beta-1,6-glucanase	Beta-1,3(1,4) glucanase	CMC –ase	Chitinase
1034.0	9.0	1482.0	328.5	38.3

Fig. 3. The effect of temperature (A) and pH (B) on activity (1) and stability (2) of beta -1,3-glucanase of *T. hamatum C-1*.

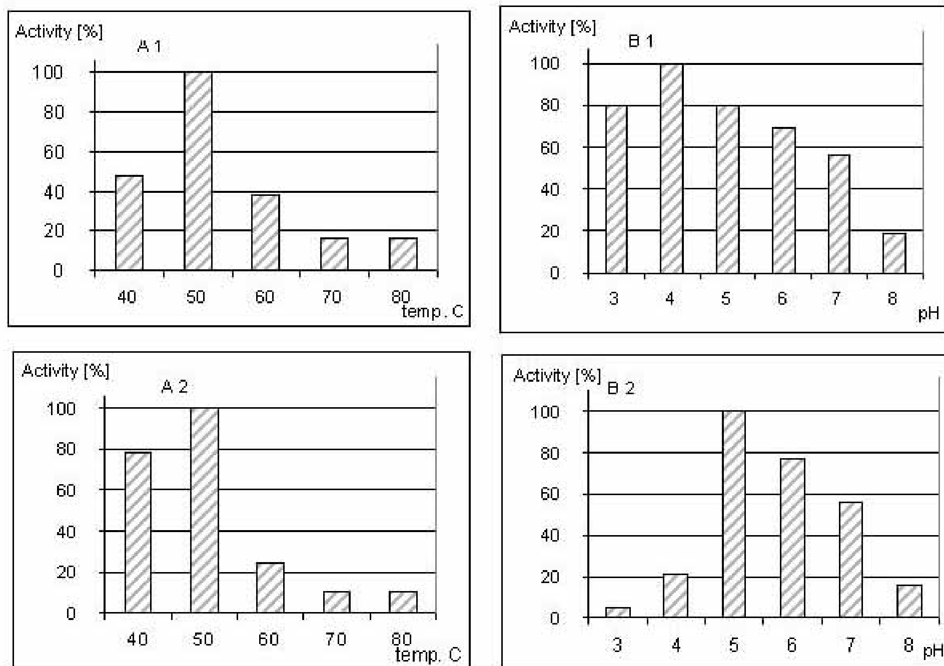


Fig. 4. The effect of various factors on beta-1,3-glucanase activity of *T.hamatumC-1*.

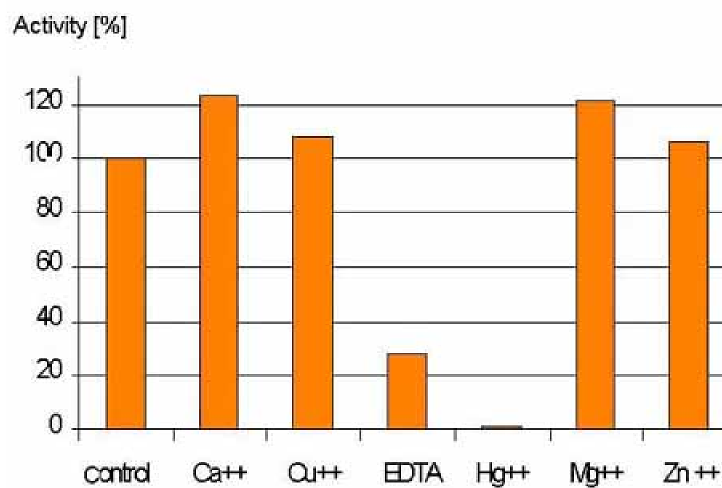


Fig. 5. Analysis of products after enzymatic hydrolysis of laminarin using HPLC.

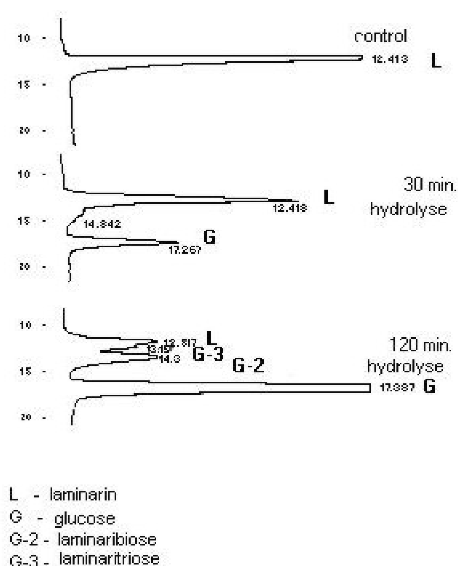


Table 2. Quantity of glucose after laminarin hydrolysis using beta -1,3- glucanase of *T.hamatum C-1.*

HYDROLYSIS TIME (min.)	QUANTITY OF GLUCOSE (%)
30	29
120	88

The enzymatic preparation obtained from *T.hamatum* C-1 was successfully used in the process of protoplast formation of *Yarrowia lipolytica* cells (70% of protoplasts after 1 h; 100% after 2 h) and of *S. cerevisiae* (60% after 2 h; 100% after 4h), (Table 3).

Table 3. The effect of enzyme preparations in protoplast formation of *Y.lipolytica* and *S.cerevisiae* cells.

TIME (h)	PROTOPLAST FORMATION (%)			
	Saccharomyces cerevisiae		*Yarrowia lipolytica*	
	SIGMA preparation	*T.hamatum* preparation	SIGMA preparation	*T.hamatum* preparation
0.5	80	0	100	30
1.0	100	0		70
2.0		60		100
3.0		80		
4.0		100		

Literature data show the different ability of yeast cells to be hydrolysed by lytic enzymes, not only among species, but also in the relation to age of cells used in protoplast formation. Kopecka [4] obtained the protoplasts of *Schizosaccharomyces versatilis* and *S.pombe* in 30 min only in the presence of a mixture of lytic enzymes from the stomach of a snail and an enzymatic preparation from the culture of *T.viride*. Evans [2] obtained after 1h 95% of protoplast of *S. cerevisiae* in the presence of Zymolase (from Arthrobacter luteus), while with enzymes from the culture of *Rhodotorula rubra* at the same time only 15% of protoplasts were obtained. In this work, the commercial preparation of lytic enzymes from Sigma company used for comparison was more effective in the protoplast formation of *Y.lipolytica* and *S.cerevisiae* (respectively 100% of protoplast after 0.5h and 100% after 1h), but the value of enzyme activity in the used dose was about twice higher in case of Sigma preparation than in case of own preparation from *T.hamatum* (Table 4) So, after some purification steps, the studied enzymatic preparation can be more effective in the protoplastisation process. The presented results suggest that the study on enzymes produced by the strain *T. hamatum* C-1 is important.

Table 4. Activities of enzyme preparations in doses used in protoplast formation.

Enzyme preparation	Activity U/30 mg of preparation	
	Beta-1.3-glucanase	Chitinase
Sigma	60.3	0.1
T.hamatum C-1	31	1.15

CONCLUSIONS

1. The strain of *Trichoderma hamatum C-1* is able to produce beta-1,3-glucanases on mineral medium enriched with fodder yeast supplemented with Avicel cellulose as well as on glucose.
2. Beta-1,3-glucanases were characterized by optimal activity at pH 3.0-5.0 and 50°C and stability at pH 5.0 and 50°C.
3. The enzyme activity was inhibited in the presence of $HgCl_2$, EDTA and stimulated by cations such as Mg^{+2} and Ca^{+2}.
4. 90% of glucose and a little laminaribiose and laminaritriose were obtained after enzymatic hydrolysis using HPLC analysis.
5. The enzymatic preparation was effective in protoplast production of Yarrowia lipolytica A101-1.31 cells (100% of protoplast after 4h) and Saccharomyces cerevisiae cells (100% after 2h).

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