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MOULDS LYTIC ENZYMES IN THE PRODUCTION OF *YARROWIA* HIGH-PROTEIN FEED ADDITIVES

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ABSTRACT

The optimal conditions for the degradation of the cell wall of *Yarrowia lipolytica* yeast, leading to the release of intracellular proteins by hydrolases of *Trichoderma* and *Aspergillus* moulds, were determinated. Hydrolysis of *Y. lipolytica* yeast biomass was performed with the use of various amounts of concentrated or non-concentrated enzymes, as well as yeast biomass subjected or not subjected to thermal inactivation. The analysis of enzymatic hydrolysis process demonstrated that the most effective was the 8-hour hydrolysis of thermally not inactivated *Y. lipolytica* yeast biomass with 6-fold concentrated enzymatic preparation, which resulted in the release of 127.86 mg of intracellular proteins per 1 g dry biomass.

Key words: chitinases, SCP, *Trichoderma*, *Aspergillus*, laminarinases.

INTRODUCTION

The search for cheap and efficient protein sources has been one of the most important issues in the field of animal husbandry. In order to solve this problem, biotechnology has offered a good source of SCP (single cell protein) such as the biomass of yeast and enzymes, to additionally increase the accessibility of protein contained in yeast cells.

Trichoderma spp. are filamentous fungi living in the soil and within the ecosystem of roots. They constitute the largest group of moulds isolated from soils of most climatic zones, where their count reaches 10^1–10^3 colony forming units per gram. It has been proven that some strains are capable of long-term colonization of the surface of plants roots and the epidermis of roots to a depth of several layers of cells, which is referred to as "rhizosphere competence". It is the ability to colonize the roots of plants and to grow on their surface [6, 7, 13].

These moulds produce a wide range of compounds, induce plant resistance to pathogens and directly participate in their active growth control. This process – referred to as biocontrol – prevents plant diseases and promotes their growth and development. A number of mechanisms are responsible for this [5, 8, 11]. It was noted, however, that mycoparasitism which is one of the most important and best-known bio-control mechanisms is based on the ability to degrade the components of pathogen cell wall with the use of the enzymes produced by *Trichoderma* [7].

The enzymes produced by the fungi, which also degrade polysaccharide cell wall components and belong to the group of cell wall degrading enzymes (CWDE), including: chitinases, β-glucanases and β-glycosidases, are involved in the process of mycoparasitism [12, 17, 23]. It is considered that the most important among them are β-1,3-glucanases due to wide spreading of β-1,3-glucan in cell walls of moulds against which these proteins show their enzymatic activity [15]. An important role is also played by chitinases and β-1,6-glucanases, although chitin and β-1,6-glucan represent a small percentage of the components of the cell wall of moulds [4, 16].

Because of the fact that the enzymes biosynthesized by the *Trichoderma* exhibit activity towards polysaccharides – present in fungal cells – an attempt was made to use these enzymes to degrade specific components of yeast cell wall, bearing in mind the

similarity in the construction of their walls. In yeast cells, two layers which constitute the cell wall are built with only four classes of macromolecules linked with covalent bonds: chitin, β-1,3-glucan, β-1,6-glucan and cell wall proteins [10]. However, with the use of yeast biomass as a potential high-protein feed additive, those components of yeast cell wall polysaccharide, which are hard to digest by animals, can significantly reduce the availability of intracellular proteins. Hence hydrolases produced by moulds can be successfully used for yeast cell wall degradation due to the presence of components, constituting substrates to these enzymes, and therefore contribute to the release of intracellular proteins.

The purpose of this research was to determine optimal conditions for the degradation of the cell wall of *Y. lipolytica* yeast [14] , leading to the release of intracellular proteins, with hydrolases of *Trichoderma* mouldsselected in terms of effective lytic properties.

MATERIALS AND METHODS

Microorganisms

Five strains of moulds: *Aspergillus cervinus* 219; *Aspergillus niger* XP; *Trichoderma citrinoviride* C 1; *Trichoderma hamatum* T2; *Trichoderma harzianum* T33, that originated from the collection of the Department of Biotechnology and Food Microbiology at the Wrocław University of Environmental and Life Sciences, as well as biomass of*Yarrowia lipolytica* 8665 UV1 yeast (from the American Type Culture Collection), whose cell wall biopolymers constitute a source of carbon and energy and induce lytic enzymes, were used in the study.

Strains were kept on agar slants at a temperature of + 4°C.

Media

Mould strains were stored on PDA culture medium, whereas the yeast strain on YM agar medium. For selection of mould strains and production of enzymatic preparation, a modified Saunders mineral medium (g/ml) was used KH_2PO_4 (0.20); K_2HPO_4 (0.15); $NaH_2PO_4 \cdot H_2O$ (2); Na_2HPO_4 (1.50); NH_4NO_3 (0.60); $NaNO_3$ (3); $MgSO_4 \cdot 7H_2O$ (0.30) enriched with dried biomass of *Y.lipolytica* yeast (20, 30, 40, 50, 60) and wheat germs (source of biologically-active substances) in the amount of 10 g/ml.

To obtain spores of filamentous fungi the PDB medium (12 g/ml) with glucose (10 g/ml) was applied.

The medium for the production of yeast biomass contained [g/ml]: bactopeptone (0.75); yeast extract YE (1); glycerin fraction (40); $(NH_4)_2SO_4$ (10); KH_2PO_4 (0.125); $MgSO_4 \cdot 7H_2O$ (0.5). Media were sterilized in an autoclave (120°C, 20 min).

Cultures of microorganisms

Selection of effective strain and proper composition of culture medium

The five mold strains were cultivated in 250-mL Erlenmeyer flasks. The medium consisted of: mineral Saunders medium (50 mL) with 1% m/v addition of wheat germs and 1, 2, 3, 4, 5 or 6% addition of *Y. lipolytica* 8665 UV1 dried biomass (inducer of lytic enzymes biosynthesis and carbon source). The cultures were inoculated with 0.5 mL of a suspension of microorganisms with a density of 108 cells/mL obtained from slant with 0.1% Tween 80 solution. A suspension of cells of a certain density was achieved with the use of Thoma chamber.

The culturing process was conducted in a rotary shaker (170 rpm) at a temperature of 25–28°C for 7 days. During that process, pH value was maintained at 5.5 with a sterile solution of 1 M NaOH and 1 M HCl. After 3, 5 and 7 days of culture, the samples were collected under sterile conditions (0.5 mL) for enzymatic activity assay.

The selection of effective strain was performed on the basis of the evaluation of enzymatic activity of chitinases and laminarinases in supernatants obtained after centrifugation (centrifugation with cooling (4°C), 5450 g, 15 min).

Production of enzymatic preparation with a selected strain was conducted as described above, but in larger flasks (500 mL) and with inducer concentration determined in the above experiments. Cultures were continued until the maximum enzymatic activity was achieved in supernatants.

The crude enzymatic preparation was obtained after centrifugation of solids from the liquid medium (centrifugation with cooling, 5450 g, 15 min).

Culture of *Y. lipolytica* yeast to produce biomass

The culture was carried out in a 3.5-L AK-3 bioreactor with a working volume of 1.1 L (after its inoculation with 30% inoculum from the shaking culture) at 30°C at stirring speed of 550 rpm and aeration rate of 1 vvm until all nutrients had been consumed. During that process, the pH value was maintained at 3.5–4.0 via automatic control with 20% NaOH solution. Once the process had been completed, the culture was centrifuged for 25 minutes at 5450 g.

Dry matter of yeast biomass was measured after it had been washed three times with sterile, distilled water and centrifuged (5450 g, 25 min) with a WPS moisture analyzer 110S by Radwag company.

Concentration of the enzymatic preparation

From the liquid obtained in the process of culturing a selected strain of moulds, as a result of solid matter centrifugation (with

cooling, 5450g, 15 min.), a crude enzymatic preparation was obtained, which was concentrated afterwards.

The process of concentration was carried out with the use of TFF Labscale ultrafiltration system with Millipore Pellicon membrane containing polyether sulphate (Pellicon XL) with MWCO of 30 kDa.

As a result of that process the following two fractions were obtained: concentrated liquid containing protein with a molecular weight over 30 kDa, and permeate containing protein with a molecular weight lower than 30 kDa.

Each of the fractions was analyzed for the activity of chitinases and laminarinases, as well as for the content of proteins.

Determination of the optimal temperature of lytic enzymes
In order to determine the optimal temperature of enzymes activity in the concentrated enzymatic preparation, the enzymatic activity of chitinases and laminarinases were determined at temperatures of: 25, 30, 35, 40, 45, 50, 55 and 60°C

Process of hydrolysis of cell walls in the biomass of *Y. lipolytica* yeast with the use of enzymatic preparation.
Enzymatic hydrolysis process was conducted in 2-mL Eppendorf's test tubes. To 0.2 g (0.039 g d. m.) of wet biomass of *Y. lipolytica* yeast (both thermally inactivated at 120°C or non-inactivated), various amounts of the enzymatic preparation were added: 0, 0.2, 0.4, 0.6, 0.8 or 1 mL of solution, and completed with acetate buffer (0.05 M, pH 4.8) to 1 mL.

The reaction was carried out in a TS-100 Thermo-Shaker by Biosan company (at temperatures optimal for the lytic enzymes, 1000 rpm) for 1, 4, 8 and 12 hours. The reaction was stopped by centrifugation (10 920 g, 10 min). The quantity of released protein was determined in the supernatant.

Determination of enzymatic activity
The activity of β-1,3-glucanase (laminarinases) and chitinases was determined according to Witkowska and Maj [22], using substrates: laminarin, and chitin respectively, by running the enzymatic reaction at 50°C, pH 5.0 and for 30 min. Reaction products, i.e. glucose in the case of β-1,3-glucanase and glucosamine in the case of chitinases were determined colorimetrically using dinitrosalicylic acid (Sigma). Activities of the analyzed enzymes were expressed as nKat/mL.

Determination of protein content in enzymatic hydrolysates by Bradford method [1].
The obtained complex of proteins with Coomassie Brilliant Blue G-250 was determined by spectrophotometry at a wavelength of $\lambda = 595$ nm. A mixture of 0.02 mL of the sample with 1 mL of the diluted Bradford reagent was intensively mixed for 10 min at room temperature. Then absorbance was measured at $\lambda = 595$ nm.

Statistical analysis. Statistical analysis was performed to support outcoming conclusions, nevertheless no relevant information has been acquired.

RESULTS AND DISCUSSION

In order to obtain enzyme preparation which would include active laminarinases and chitinases, necessary for the degradation of cell walls of yeast, potential bio-synthesizers of these enzymes – *Trichoderma* and *Aspergillus* strains – were cultivated in this study.

The ability of *T. citrinoviride* C1*, T. hamatum* T2*, T. harzianum,* T33, *A. cervinus* 219 and *A. niger* XP to produce lytic enzymes, such as chitinases and laminarinases, was shown in shaking cultures in the presence of yeast biomass as a carbon source and anr of the biosynthesis of enzymes. While analyzing the individual cultures of moulds, differences were observed in the levels of the activity of the tested enzymes depending on both the amount of added yeast biomass and incubation time (Fig. 1 and 2).

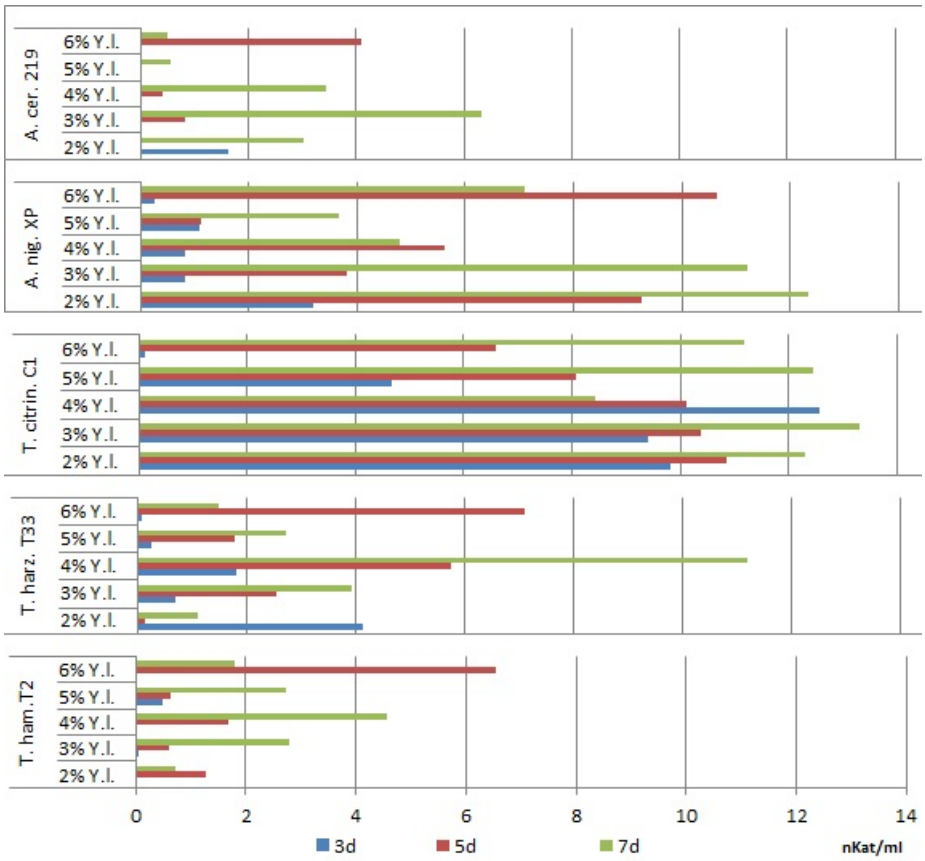

Fig. 1. Dynamics of laminarinases biosynthesis by filamentous strains tested depending on *Yarrowia lipolytica* biomass addition to the culture

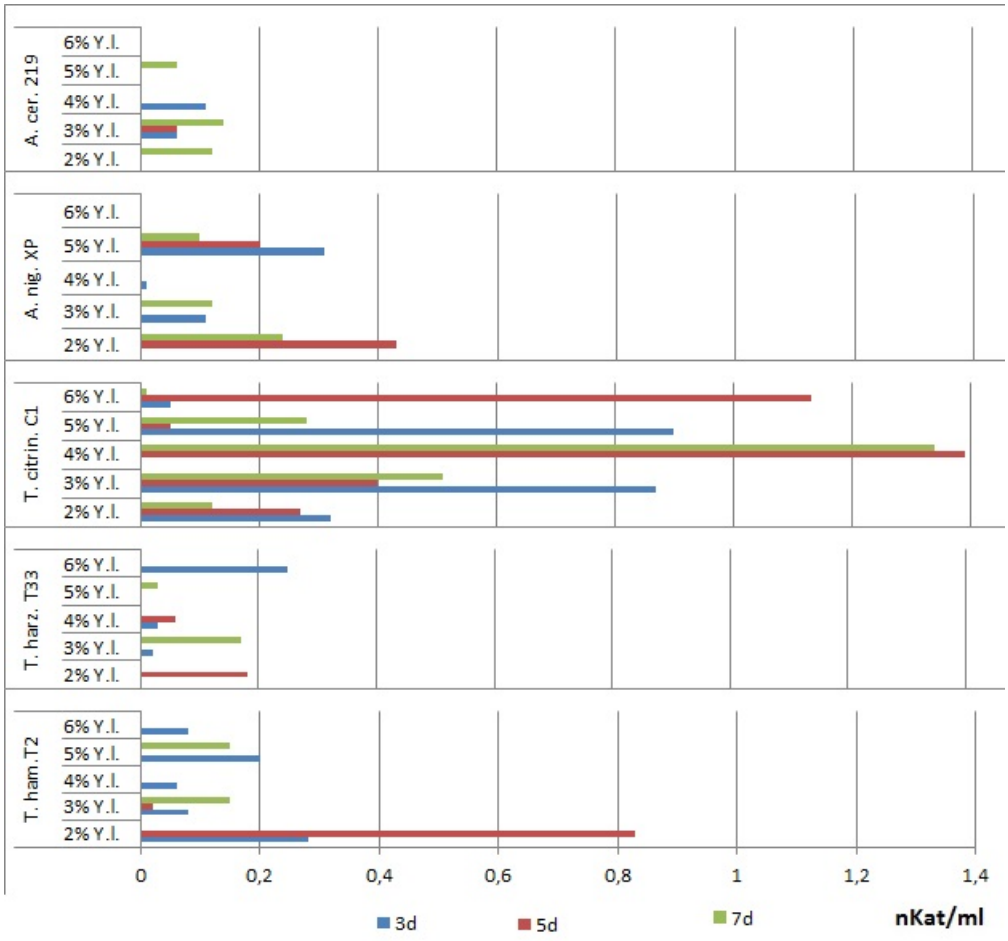

Fig. 2. Dynamics of the chitinases biosynthesis by filamentous strains tested depending on *Yarrowia lipolytica* biomass addition to the culture

In general, all tested strains showed greater ability to synthesize laminarinases than chitinases, with activities of 6.32–13.22

nKat/mL and 0.14–1.38 nKat/mL (maximum values for a given strain), respectively.

Trichoderma citrinoviride C1 turned out to be effective in the biosynthesis of both laminarinases as well as chitinases in cultures with 4% addition of yeast biomass laminarinase activity was 12.57 nKat/mL at day 3 and chitinase activity 1.39 nKat/mL atas day 5 of the culture. The remaining tested strains showed the maximum activity at a similar (e.g. laminarinases, T33) or significantly poorer level compared to the C1 strain. In the case of the C1strain, it was observed that the secretion of extracellular laminarinases (in nKat/mL) was approximately at the same level (12.34–13.23–12.57–12.47 nKat/mL) in all cultures, with different levels (2%–3%–4%–5%) of yeast biomass used as carbon add the.

In the case of *Aspergillus* strains, the maximum secretion of laminarinases was reported in the cultures with 2 or 3% addition of yeast biomass, mostly on day 7 of culture, whereas in the case of *Trichoderma* strains – in cultures with 4 or 6% yeast biomass addition, and in most cases on day 7 of the culture (Fig. 1).

Concerning chitinases, (as judged by the maximum values obtained in cultures), strain C1 produced chitinase activity at 1.5 to 5 times higher level than the other tested strains of *Trichoderma* and at 3 to 10 times higher level in comparison to the strains of *Aspergillus* (Fig. 2). In the majority of tested strains, the maximum activity of chitinases was measured in the 3–5 day of incubation, i.e. slightly earlier than in the case of laminarinases (7 day).

In the presented study, the hydrolysis process of cell walls of *Y. lipolytica* yeast with crude C1 preparation (C1 supernatant) was shown, which was characterized by the highest activity of laminarinases and chitinases among the tested strains.

The culture supernatant was subjected to the process of ultrafiltration– with the use of membrane stopping particles with a molecular weight of > 30 kDa and allowing particles with < 30 kDa – for its concentration and partial purification from ballast proteins. As a result, a 6-time greater liquid density was obtained. The fraction > 30 kDa was referred to as concentrated preparation C1 (Fig. 3).

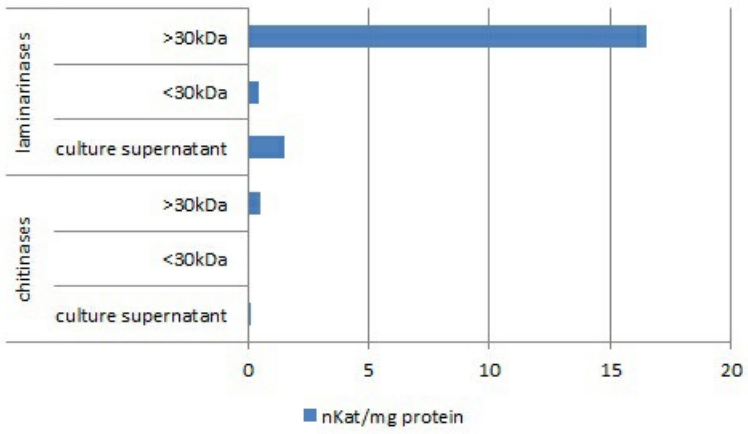

Fig. 3. Protein separation in the ultrafiltration process of C1 post-culture liquid

In the < 30 kDa fraction chitinase activity was not observed whereas laminarinase activity was low (2.5% compared to the activity in the fraction > 30 kDa). Both activities were found in the fraction of > 30 kDa, which proves that the analyzed enzymatic proteins are molecules with molecular weight over 30 kDa.

Thus, as a result of the 6-fold concentration of supernatant with the ultrafiltration method (with the use of MWCO membrane = 30kDa), 5 times higher degree of purification of chitinases was achieved, as well as nearly 11 times higher degree of laminarinases purification (Tab. 1, Fig. 3).

Table 1. Specific activity and purification level of chitinases and laminarinases of C1 culture and concentrated preparation

Activityand purification	chitinases		laminarinases	
	in culture	concentrated	in culture	concentrated
Specific activity [*nKat*/mg protein]	0.10	0.50	1.50	16.48
Purification level	1	5	1	11

A research by El-Katatny et al. [2, 3] showed that the preparation produced upon ultrafiltration (MWCO = 30 kDa) of supernatant from the culture of *T. harzianum* Rifai T24 strain allowed 25% inhibition of growth of *S. rolfsii* phytopathogen (even in 10 x dilution). While concentrating the liquid with the use of MWCO 10kDa membrane, a very weak inhibition effect had been observed, which indicated the presence of a lytic enzyme solely in the MWCO = 30 kDa fraction, which was also confirmed in the presented research.

In regard to the application of enzyme preparation in degradation of cell walls of phytopathogens, the optimum temperatures for laminarinases and chitinases were determined in the analyzed preparation. Within the tested range of temperatures 25–60°C, the optimum temperature was at 40°C for chitinases and at 50°C for laminarinases (Fig. 4 and 5).

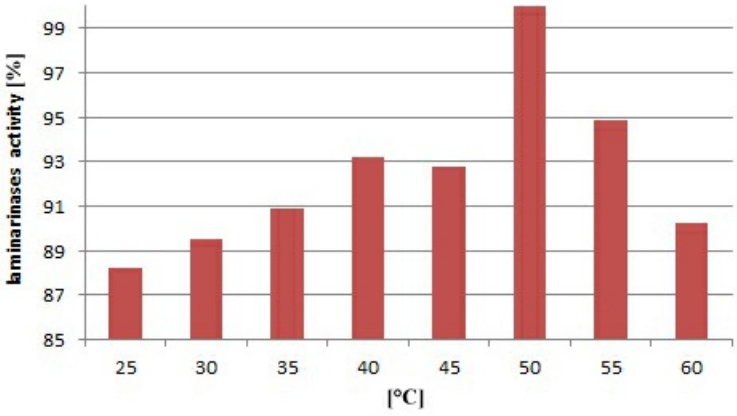

Fig. 4. Activity of laminarinases in condensed preparation at different temperatures

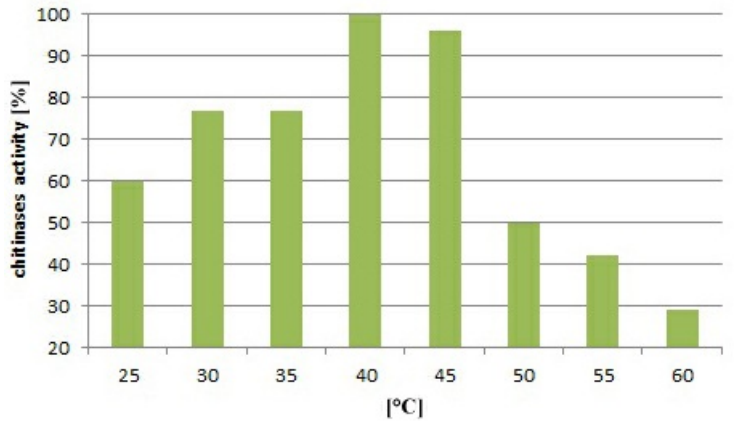

Fig. 5. Activity of chitinases in condensed C1 preparation at different temperatures

Similar results were obtained by El-Katatny et al. [3] who examined the contribution of*T. harzianum* Rifai T24 strain in eradication of *S. rolfsii* phytopathogen by enzymatic degradation of cell walls, where maximum chitinase activity of 0.05 *JCh* was achieved at 40°C and that of laminarinase reaching 11.2 *JL* at 50–60°C. In turn, in studies conducted by Kapata and Panda [9] the best results of the maximum activity (0.96 *JCh*) of chitinases biosynthesized by *T. harzianum* NCIM 1185 strains was obtained upon the enzymatic reaction at a much lower temperature, i.e. 23°C.

The enzymatic hydrolysis of thermally inactivated and not inactivated *Yarrowia lipolytica* yeast biomass was conducted with C1 enzymatic preparation. The effect of the thermal inactivation on the hydrolysis was evaluated based on the quantity of proteins released from cells determined with Bradford method (Fig. 6).

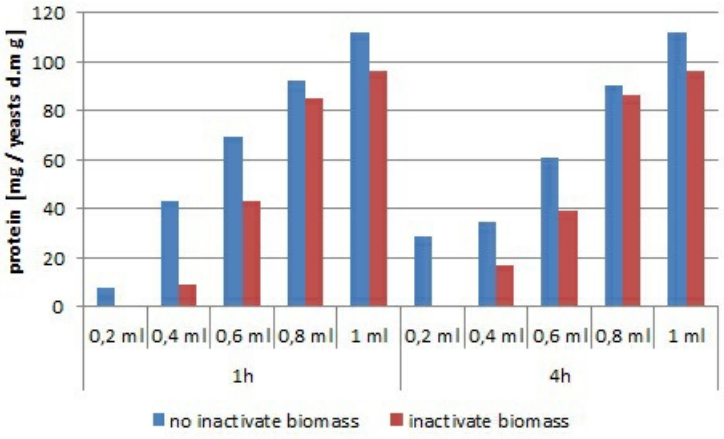

Fig. 6. Quantity of released protein per 1 g of dry matter of yeast, with or without thermal inactivation, depending on the concentration of C1 preparation used and time of hydrolysis process

The results obtained indicate that apart from increasing quantities of enzymes (0.2–1 ml) in enzymatic reaction, a better effect of protein release from *Y. lipolytica* yeast biomass was observed, especially in case of thermally not inactivated biomass. After 4 hours of enzymatic reaction, 111.86 mg of protein were released per 1 g d. m. yeast with 1 mL of the preparation having the activity of 110 nKat/mL (Fig. 6). However, in the case of thermally inactivated yeast biomass used in the process of hydrolysis

with the highest concentration of the same specimen, only 96.4 mg of protein per 1 g d. m. were obtained after 4 hours hydrolysis.

Few published papers deal with the application of lytic enzymes in the release of intracellular proteins. These include the research by Revah-Moiseeva and Carrod [19], where attempts were made to use chitinases for the utilization of chitinous waste and cellulose bioconversion to obtain microbial proteins (SCP-single cell protein). According to these authors, chitinase enzymes obtained in *Serratia marcescens* culture were used for hydrolysis of chitin and biomass of *Pichia kudriavzevii* yeast which was the source of SCP. In this case, protein release reached 45%.

Other sources [21], indicate that in order to obtain the SCP with the use of chitinases, the following types of yeast and moulds could also be used: *Hansenula polymorpha, Myrothecium verrucaria, Candida tropicalis* and *Saccharomyces cerevisiae*. Vyas (1991) [20, 21] used in their research *M. verrucaria* chitinases for chitin degradation and for obtaining SCP from *S. cerevisiae* biomass and achieved satisfactory results at 61% of free protein. This is due to a high activity of *N*-acetylglucosaminidases, which was demonstrated by a significantly high level of *N*-acetylglucosamine determined in the culture medium.

Most of the published papers, concerning *Trichoderma* spp. moulds and enzymes bio-synthesized by them, mainly focus on their involvement in the mycoparasite process. Numerous studies concentrated on the use of these enzymes in yeast cell protoplastization.

Xiao and Morell [25] have studied the use of *Trichoderma harzianum* enzymes in the protoplast formation of the germinative *Ophiostoma picea* cells, causing major timber defect – wood discoloration. Witkowska et al. [23, 24] used enzymes of *Trichoderma reesei* M7-1 in the *Y. lipolytica* and *Schwaniomyces occidentalis* yeast protoplast formation andachieved satisfactory results in the process. Another example of the application of lytic enzymes of moulds is described by Rattanakita et al. [18] use of chitinases biosynthesized by *Aspergillus* sp. S1-13 strain for waste disposal in the form of chitinous shells of shrimps. Chitin-lytic activity of this strain was observed only in the solid-state culture of waste received after shellfish processing. After selecting the optimum composition of the substrate and parameters, chitinases activity increased 3.5-fold. Studies have enabled selecting the effective strain for degradation of waste from shrimp and their processing into chitin.

Studies on the release of intracellular proteins from yeast biomass using enzymatic preparation obtained after the fungus culture are innovative and are to be continued, because there is still a lot of issues that need to be elucidated, and this process deserves special mention, since it affords the possibility of obtaining high-protein and easily-digestible feed additive at low cost, without the use of chemical reagents detrimental to the natural environment.

CONCLUSION

Of the five tested microorganisms, the strain of *T. citrinoviride* C1 turned out to possess the best lytic properties against cell was biomass of *Y. lipolytica* yeast. The most favorable conditions for the cultivation of *T. citrinoviride* C1 strain included 5-day culture with 4% addition of *Y. lipolytica* yeast biomass as a carbon source. Enzymatic hydrolysis of *Y. lipolytica* yeast biomass to thermal inactivation gave better results – in the form of intracellular protein – than hydrolysis of heat-inactivated biomass.

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