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Microbiology

Selection of Microscopic Fungi-Proteases Producers

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ABSTRACT. **Among the collection of microscopic fungi isolated from different ecological niches of Southern Caucasus available at Durmishidze Institute of Biochemistry and Biotechnology, fungi strains – active producers of protease were selected by screening under deep cultivation conditions. Among the producers of proteases the most active were representatives of the following genera – *Aspergillus*, Penicillium, *Mucor*. Active producers of protease – *Penicillium* sp. To 1-10 (moderate-halophile), *Mucor* sp.T 44 (thermotolerant), *Aspergillus* sp. To 1-3 (alkalitolerant) were chosen. Myco-toxicological studies showed that the selected strains were neither toxic nor pathogenic. Through study of physiological and biochemical characteristics of the selected strains under deep cultivation conditions the nutrient media were optimized and the optimal conditions (temperature, pH) were determined. As a result of determining optimal conditions and optimization of nutrient media components, the activities of proteases produced by the strains were increased by 22-112%. Enzyme preparations were received and temperature and pH optimums for activity of protease, produced by selected strains, determined as a result of conducted study.**

Key words: *microscopic fungi, strain, protease, moderatehalophile, thermotolerant, alkalitolerant.*

Development of biological disciplines over the recent years and several researches carried out in the areas of molecular biology, molecular genetics and genetic engineering required to create new microbial collections. The existing and new collections enable to detect strains for industrial purposes, which will be used for creation of precious metabolites of microbial origin. Detection and production of enzyme preparations of microbial origin is especially important.

Production of enzyme preparations occupies one of the leading positions in modern biotechnology and belongs to those branches of industry production, volume of which is permanently growing and the areas of application are expanding.

Among the microbial enzymes, protease is an important enzyme usable in food and light industries [1]. Receiving the preparations of high active protease enzyme is possible by detection of the active protease producers from the strains existing in collection of microorganisms.

During the last 10 years, selection of microorganisms has clearly demonstrated that the search for stable forms of enzymes is appropriate mainly among those microorganisms that exist within the relatively critical conditions [2].

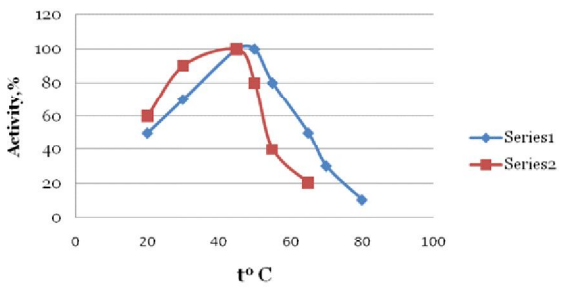

Fig. 1. The effect of temperature on biosynthesis of proteases.
Strains: 1. *Mucor* sp.T 44 2. *Aspergillus* sp. To 1-3

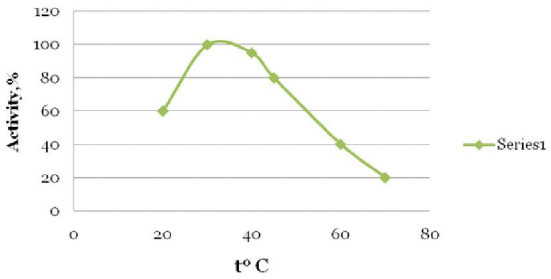

Fig. 2. The effect of temperature on biosynthesis of proteases.
Strain: *Penicillium* sp. To 1-10

Materials and Methods

Research objects were selected among microscopic fungi cultures from different ecological conditions.

At the initial stage strains were grown and developed on solid nutrient medium, beer wort agar of the following composition (per l): 0.5 l beer wort 7° B, 0.5 ordinary water, 20.0 g agar-agar. pH value (pH 5.5-6.0) was brought to desirable indication by adding alkali – 1 M NaOH (when pH was low) and by adding acid – 1 M HCl (when pH was high).

Nutrient medium poured in flasks was sterilized during 40 minutes at 0.7 atm. Flasks were placed in thermostat, in which temperature corresponded to the optimal growth temperature required for each strain. Simultaneously, optimal temperature and pH needed for growth and development of grown and identified strains were established, the influence of several concentrations of NaCl on growth and development of cultures was studied. Temperature and pH optimums were established to be of maximal growth of fungi cultures that was defined by colony diameter and growth speed.

In order to reveal haloophilic (tolerant to high concentration of NaCl) cultures, different concentrations of NaCl from 0.5 M to 4.0 M (2.93%-23.2%, respectively) were added to the initial nutrient medium. Screening of enzyme producers under deep cultivationwas conducted.10-day conidia culture suspension served as cultivation material. Deep cultivation of certain strains of microscopic fungi was carried out in 750 ml Erlenmeyer flasks in a thermostatic shaker (180-200 rpm) at 30°C for 72-80 hours.

In order to obtain protease, deep cultivation in liquid nutrient mediumof the following composition (in %): $KN0_3$ – 0,1; KH_2PO_4 – 0.1; $MgSO_4 \cdot 7H_2O$ – 0.007; KCL – 0.05; $FeSO_4 H_2O$ – 0.005; yeast extract – 0.5, casein 0.1; pH 5.0 was conducted.

To determine protease activity Anson's modified method was applied [3]. After cultivation, cultural liquid was centrifuged at 4000 rpm. Ferment activity was defined in centrifugate of cultural liquid; as it was established by preliminary tests, ferments were concentrated mainly in cultural filtrate and only 5-10 % was left in biomass.

Pathogenicity and toxicity of protease revealed among microscopic fungi cultures were investigated as well.

Zoopathogenicity was investigated by intravenous injection of fungal suspension in experimental rabbits [4]. The Berestetsky's method [5] was used to establish phytopathogenicity. Toxicity was studied by Diekman method [6].

Nutrient medium for protease producers was optimized via screening and optimal conditions for deep cultivation of protease producers were established. Casein, cottage cheese, soya, disaccharides and some monosaccharides, mainly, maltose, lactose, manite, saccharose, ramnose, sorbite, xylose, galactose, glycerin and cellobiose – by 0.8 % of carbon were selected as a carbon source.

The following salts as mineral source of nitrogen: sodium nitrate and potassium nitrate, ammonium sulphate, ammonium nitrate and double-substituted ammonium phosphate, mono- and double-substituted

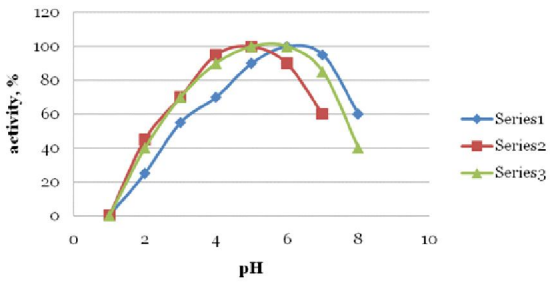

Fig. 3. The effect of pH on biosynthesis of proteases. Strains: 1. *Aspergillus* sp. To 1-3; 2. *Mucor* sp. T 44; 3. *Penicillium* sp. To 1-10

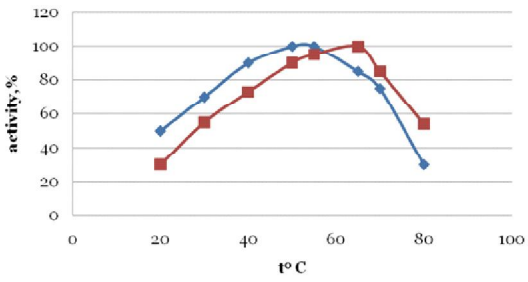

Fig. 4. The effect of temperature on protease activity. Strains: 1. *Aspergillus* sp. To 1-3 2. *Mucor* sp.T 44

potassium phosphate were used.

While receiving technical preparation the following scheme was used: culture liquid obtained from deep cultivation of cultures was filtered and cooled to 4^0C; then different quantities of organic solvents: acetone, ethyl alcohol and isopropanolewere added.

In order to define temperature optimums for activity of enzyme preparation, enzyme activity was measured between 20ºC to 80ºC at 5ºC intervals. In order to define pH, pH of incubation medium was changed from pH 2.0 to pH 10.0 at 5.0 intervals. Activities were determined by standard methods and expressed in percents.

Results and Discussion

Microscopic fungi were selected among the cultures from different soil-climatic zones. In order to choose active producers of protease, screening under deep cultivation conditions was carried out.

Resulting from the studies the protease producers were revealed mainly among the representatives of the genera:*Aspergillus, Mucor* and *Penicillum*. Most of them were thermotolerant, alkalitolerant and halophile. Finally, the following three cultures –*Penicillium* sp. To 1-10 (moderate-halophile), *Mucor* sp.T 44 (thermotolerant)and *Aspergillus* sp. To 1-3(alkalitolerant) were chosen for further experiments. After micotoxic examinations, it was found that the cultures were not pathogenic and toxic allowing them to be applied in industry.

Proper selection of food areas plays significant role under optimal conditions of microbes' activity and supports production of enzymes in great quantities [7,8]. Carbon source was selected for optimization of optimal nutrient medium for protease producer microscopic fungi strains. The most intensive increase of protease activity was caused by casein in *Aspergillus* sp.To 1-3 and *Mucor* sp.T44 and by soybean flour– in *Penicillium* sp.To 1-10.

It was established that almost all selected strains absorb peptone, ammonium sulphate $(NH_4)_2SO_4$ from carbon sources, but they reveal maximal activity when using potassium nitrate. It was found that proper selection of potassium nitrate concentration is also important. 0.25-5.00 g of ammonium sulphate was added to the nutrient medium g/l. Nutrient medium without nitric source was served as a control.Selected 1.0g appeared to be optimal.

Monosubstituted potassium phosphate occurred to be optimal addition to the nutrient medium for the purpose of production of enzymes by strains.

In further studies, nutrient mediums, in which the mentioned strains produced maximal amount of protease under deep cultivation, were used:

1. Composition of liquid medium for protease production, %: $KN0_3$ – 0.1; KH_2PO_4 – 0.1; $MgSO_4 \cdot 7H_2O$ –0.007; KCl – 0.05; $FeSO_4 \cdot H_2O$ – 0.005; yeast extract – 5.0; casein –1.0.

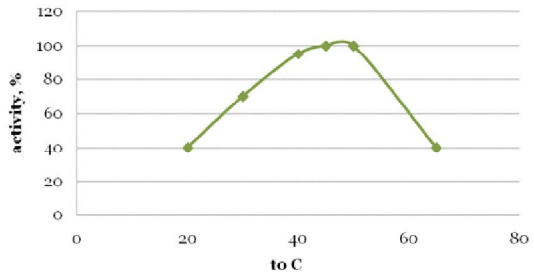

Fig. 5. The Effect of temperature on protease activity. Strain: *Penicillium* sp. To 1-10

Table 1. pH optimums of proteases activity

Producers	pH-Optimum
*Penicillium*sp. To 1-10	8.5
*Aspergillus*sp. To 1-3	6.5
*Mucor*sp.T 44	4.4

2. Composition of liquid medium for protease production %: Soy bean flour – 3.0; Na_2HPO_4 – 1.5; $KN0_3$ – 0.2; KCl – 0.05; $MgSO_4$ – 0.015

After studies, the quantity of enzymes produced by these cultures was increased, thus, allowing these enzymes to be used in different spheres of industry.

Life ability of microorganisms greatly depends on environmental conditions.

Temperature is the most important physical factor that affects the growth and development of microorganisms [7]. Considering optimal growth temperature of active producers of protease, deep cultivation was correspondingly carried out at different temperatures, namely at above 30ºC to 55ºC at 5ºC intervals.

It was found that *Mucor* sp. T44 reveals high protease activity at 40ºC, *Aspergillus* sp. To 1-3 – at 35ºC and *Penicillium* sp.To 1-10 – at 30ºC (Fig 1, 2). pH also has great impact on physiological activity of microorganisms. In this case, maximal pH of growth for the mentioned cultures was considered [9]. Optimal pH was 5.5, 7.2 and 6.2, respectively (Fig.3). Finally, optimal cultivation conditions and nutrient medium for active producers of protease, resulting in increase of activities of the enzymes by 20-112% were established.

At the next stage, technical preparations of proteases produced by the mentioned strains were obtained and it was proved that ethyl alcohol is the best organic solvent.

Temperature optimums for protease activity of strains were determined (Figs. 4, 5).Optimal temperature for protease activity of *Mucor* sp.T44 is 70ºC, *Penicillium* sp. To 1-10 – 50 ºC and *Aspergillus* sp.To – 60 ºC.pH optimums of enzymes produced by these cultures were studied. pH of incubation medium varied between 2.0 and 10.

The results obtained are shown in Table 1, from which can be seen that pH optimum of protease produced by the strains corresponds to optimal pH of their deep cultivation. Technical preparation of the obtained protease allows not only widening their range, but enables its application in medicine as a means for facilitating digestion and treating severe wounds. Protease, together with amylases, can be used in bread and milk production, as well as in textile industry for leather processing and as an ingredient in production of detergents.

მიკრობიოლოგია

პროტეაზას პროდუცენტ მიკროსკოპული სოკოების სელექცია

ლ. ქუთათელაძე*, ნ. ზაქარიაშვილი*, მ. ჯობავა*, თ. ურუშაძე*, რ. ხვედელიძე*, გ. კვესიტაძე**

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დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტის კოლექციაში არსებულ მიკროსკოპულ სოკოებს შორის, რომლებიც გამოყოფილია სამხრეთ კავკასიის სხვადასხვა ეკოლოგიური ნიშებიდან სკრინინგის გზით, სიღრმული კულტივირების პირობებში შერჩეულია სოკოების შტამები –პროტეაზების აქტიური პროდუცენტები. პროტეაზას პროდუცენტებს შორის განსაკუთრებით მაღალი აქტივობებით ხასიათდებოდნენ *Aspergillus*-ის, *Penicillium*-ისა და *Mucor* -ის გვარის წარმომადგენლები. შერჩეული იქნა პროტეაზას აქტიური პროდუცენტები: *Penicillium* sp.To 1-10 (ზომიერი ჰალოფილი), *Mucor* sp. T44 (თერმოტოლერანტი), *Aspergillus*-sp. To 1-3 (ალკალიტოლერანტი). აღნიშნული შტამების ფიზიოლოგიური და ბიოქიმიური თვისებების შესწავლით, სიღრმული კულტივირების პირობებში მოხდა საკვები არეების ოპტიმიზაცია და ოპტიმალური პირობების (ტემპერატურა, pH) დადგენა. დადგენილ ოპტიმალურ პირობებში საკვები არეების შემადგენელი კომპონენტების ოპტიმიზაციით შტამების მიერ წარმოქმნილი პროტეაზას აქტივობები გაზრდილია 20-112%-ით. მიღებულია ფერმენტული პრეპარატები და ჩატარებული კვლევების შედეგად დადგენილია აღნიშნული შტამების მიერ წარმოქმნილი პროტეაზების მოქმედებების ტემპერატურული და pH ოპტიმუმები.

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