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Microbial Alkaline Proteases Isolated from South Caucasus

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Abstract: Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes [1]. Protease stability to high temperature and alkaline conditions is of great value for their effective application as detergents and in leather industries [2]. Active producers of proteases were selected by screening under deep cultivation conditions among the collection of mycelial fungi at S.Durmishidze Institute of Biochemistry and Biotechnology, isolated from different ecological niches of Caucasus. To determine protease activity Anson's modified method was applied [3]. Through the study of physiological and biochemical characteristics of the selected strains, in the conditions of deep cultivation the nutrient media were optimised and the optimal conditions (temperature, pH) were determined. As a result of optimization of nutrient medium the activities of proteases produced by strains were increased by 22-50%.Technical preparations of proteases were obtained by precipitation with $(NH_4)_2SO_4$ (70g/100ml). In order to define temperature optimums for protease enzyme preparations, enzyme activity was measured between 20-80^0C with 5^0 intervals. In order to define pH optimums of enzyme activity, pH of incubation medium was changed from 2.0 to12.0, by pH 0.5 intervals. Optimum temperatures of action of protease preparations from three producers - *Mucor sp.*T-44, *Penicillium sp.*T 1-10 and *Aspergillus sp*. 1-3 were estableshed to be within the range 60-70°C. Especially considerable are preparations from alkaliphile *Aspergillus sp* P-39 with pH optimum of action at pH 10.5 and *Penicillium sp.* 1-9 with pH optimum of action at pH 11.5 Performed experiments revealed that selected preparations of proteases can be successfully used in different branches of industry.

Keywords: mycelial fungi, strain, protease, enzyme, alkaliphile, temperature optimums, incubation medium

Introduction

The basic problems use of enzymes in the industry are high cost and low stability of the great majority of commercial enzymes far not always satisfying the requirements of industrial processes. Availability of stable forms of these enzymes would not only expand area of their application, but also reduce the price of products produced by the use of these enzymes. Receiving of enzyme preparations occupies one of the leading positions in modern biotechnology and belongs to those branches of industry whose production volume is permanently growing and the areas of application is expanding. Among the microbial enzymes, important enzyme usable in food and light industries are Protease.[4] Receiving of the high activity Protease enzyme preparations is possible by detection of the active Protease producents from the strains existing in collection of microorganisms. Molecular genetics and Genetic engineering have required to create new microbial collections. The existing and new collections allow possibility to detect the industrial purpose strains which will be used for creation of precious metabolites of microbial origin. Detection and receiving of microbial origin enzyme preparations is specially important. During the last 10 years, selection of microorganisms has clearly demonstrated that the searching for stable forms of enzymes is appropriate mainly among those microorganisms that exist within the relatively critical conditions [5].

Alcalophyles are the unique microorganisms which possess both high microbiological potentials. The rescarch is essentially activated in the resent times and was directed, mainly, to the detection of the new aspects of the alcalophylic microorganisms use different branches of microbiology and biotechnology including enzymology.

New possibilities of the alkaline proteases, amylases and cellulases produced by the said microorganisms use at the technological scale were studied. A lot of pwpers which concem the alkaline proteases extracted from alcalophylic microorganisms.[6].

The proteases are widely used in different branches of industry. They are applied in the processing of the animal origin (in butchery, dairy, light industry, fermentative processes of skin- snd fur-processing branches,bakery, medicine, chemistry, washing products, etc.) From this point of view the search of the new microoganisms' strains-the producents of highly active proteases is significantly actual.The scope of the present work was the extraction of the alcalofylic proteases from different soil-climatic zones of Georgia and search of the strains with high protease activity among them.

Materials and Methods

The soil probes were taken in the following regions of Georgia; Racha region (raw humus calcareous), subalpine zone – Kazbegi region (mountain-meadow soils), humid subtropical climate zone – Poti region (lowland bog soil and podzols), steppe zone – Sighnaghi region (chernozem and chestnut soils), semi desert zone – Marneuli region (chestnut, alkalized and nitric soils), dry subtropical zone – Telavi region (brown soil, chernozem, alluvial soil), continental climatic zone – Borjomi region (brown forest podzolized, volcanic soils).

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

Nutrient medium poured in flasks were sterilized during 40 minutes at 0.7 atm. Flasks were inserted into 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, influence of NaCl several concentrations on the 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 hemophilic (NaCl high concentration) cultures, we added NaCl of different concentrations from 0.5M to 4.0 M (correspondingly 2.93%-23.2%) to the initial nutrient medium.*We conducted screening of enzyme producers under deep cultivation. 10-day conidia culture suspension served as the cultivation materal.* Deep cultivation of certain strains of microscopic fungi was carried out in 250 ml Erlenmeyer flasks, in a thermostatic centrifuge (180-200 rotates/min) at 30^{O}C during 72-80 hours. In order to obtain protease , we conducted deep cultivation in liquid nutrient medium of the following composition: %: $KN0_3$-0,1; KH_2PO_4-0,1; $MgSO_4.7H_2O$-0,007; KCL-0,05; $FeSO_4.H_2O$ -0,005; yeast extract – 0.5, casein 0.1; pH 5.0

Anson *modified method was applied for defining protease activity [7]. After cultivation, we centrifuged cultural liquid at 4000 rot/min speed. We defined ferment activity in the centrifuge of cultural liquid, as established by preliminary tests, ferments are concentrated mainly in cultural filtrate and only 5-10 % is left in biomass.*

We investigated pathogenicity and toxicity of protease revealed among microscopic fungi cultures on. Zoopathogenicity was investigated by intravenous injection of the fungal suspension in experimental rabbits [8]. The method of Berestetsky [9] was used to establish phytopathogenicity. Toxicity was studied by Diekman method [10].

When obtaining technical preparation, we used following scheme : cultural liquid obtained from deep cultivation of cultures was filtered, then cooled to 4^{O}C and added with (to) different quantity of organic solvents: acetone, ethyl alcohol and isopropalone. In order to define temperature optimums for ferment preparation action, we measured ferment activity at from 20 ^{O}C to 80 ^{O}C with 5^{O}C intervals. In order to define pH, we changed incubation area pH from pH 2.0 to pH 10.0 with 5.0 intervals. Activities were defined with standard methods and expressed in percents.

Results and Discussion

Microorganisms growing under extreme of conditions often are capable to produce enzymes exceeding in stability currently used ones [11,12,13] Investigation of microorganisms-extremophiles of different groups of fungi allows selection of strains producing well balanced stable molecules of cellulases, expressing increased resistance against different critical conditions that are highly demanded in a number of industrial processes [14,15,16]. Special interest attracts thermophilic/thermotolerant fungi having potential of growth above 40^{0}C. Microscopic fungi were_chosen among cultures from different soil-climatic zones. We carried out screening under deep cultivation terms in order to choose active producers of protease.

Table 1.Protease activity of cultures from different soil-climatic zones of Georgia.

No	Culture	Proteases activity U/ml	Characterization
1	*Aspergillus sp.* K 6-11	1,6	Moderate halophile
2	*Aspergillus sp.* T 1-3	2,76	Thermophile
3	*Aspergillus sp.* N2-5	0.83	Alkalitolerant
4	*Aspergillus sp.* Av 10	0.80	Thermophile
5	*Aspergillus sp.* Sh 86	1.16	Alkalitolerant/Thermotolerant,moderate halophile
6	*Mucor sp.*T-44	2,87	Thermophile
7	*Mucor* sp K 6-3	0.40	Mesophile
8	*Mucor* sp. Ar 6	0.85	Mesophile
9	*Mucor* sp. Sh 81	0.50	Thermotolerant
10	*Penicillium* sp. K 1-7	1.20	Moderate halophile
11	*Penicillium* sp. Sh.60	0.90	Mesophile
12	*Penicillium* spTn 1-2	-	Mesophile
13	*Penicillium* sp.Tn 2-3	0.50	Mesophile
14	*Penicillium* sp. Av 1	1 34	Alkalitolerant
15	*Penicillium* sp. Ar 12	0.76	Thermophile
16	*Penicillium* sp. Sy 41	0.86	Alkalitolerant
17	*Penicillium sp.*T 1-10	3.32	Thermophile
18	*Penicillium sp.* 1-9	3.25	Alkaliphile
19	*Aspergillus sp* P-39	2,65	Alkaliphile
20	*Aspergillus* sp. L 4-0	0.96	Thermotolerant
21	*Aspergillus* sp. V 2-1	0.80	Mesophile
22	*Aspergillus* sp. J1-3	0.60	Mesophile

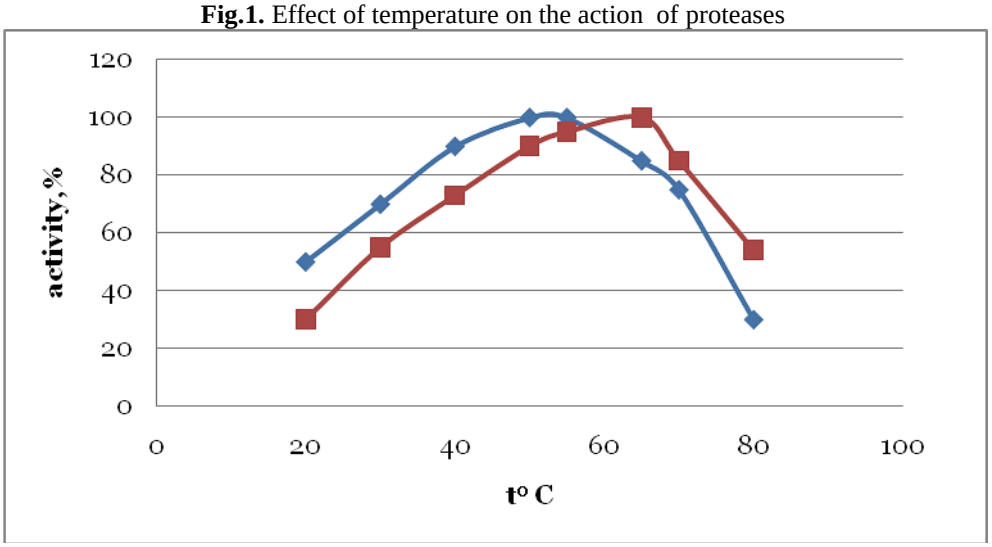
Resulting from the studies, protease producers mainly were revealed among the representatives of Aspergillus, Mucor and Penicillum genera. Most of them were thermophilic, alkaliphile and halophile.

Finally for further experiments, we chose the following three thermophilic cultures: Penicillium sp. T 1-10; Mucor sp.T- 44 and Aspergillus sp. T 1-3 ,and alkaliphile- Aspergillus sp P-39 ,Penicillium sp. 1-9

At 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.

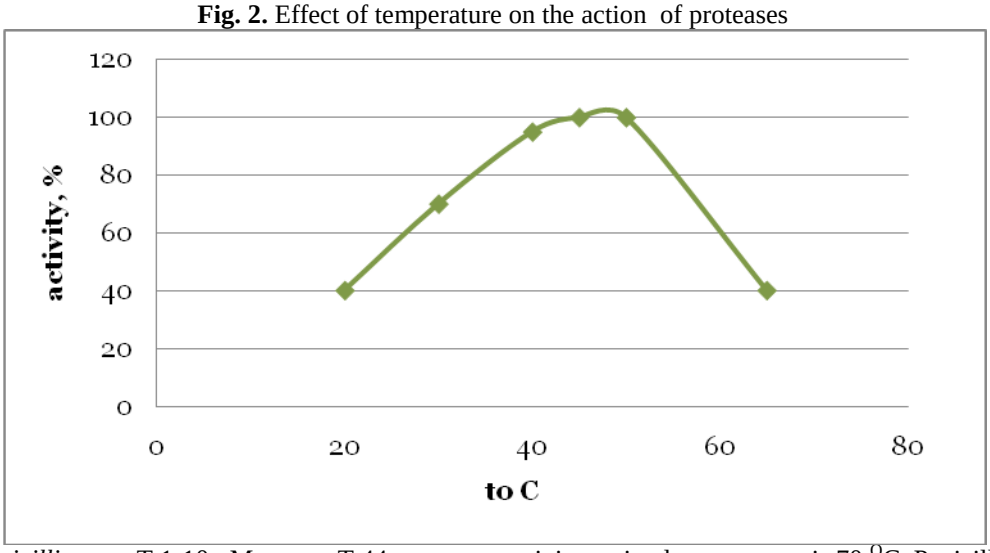
Temnperature optimums for protease activity of strains were established (Fig. 1;2,)

Fig.1. Effect of temperature on the action of proteases



Strains: 1. *Aspergillus* sp. T 1-3
2. *Mucor* sp.T 44

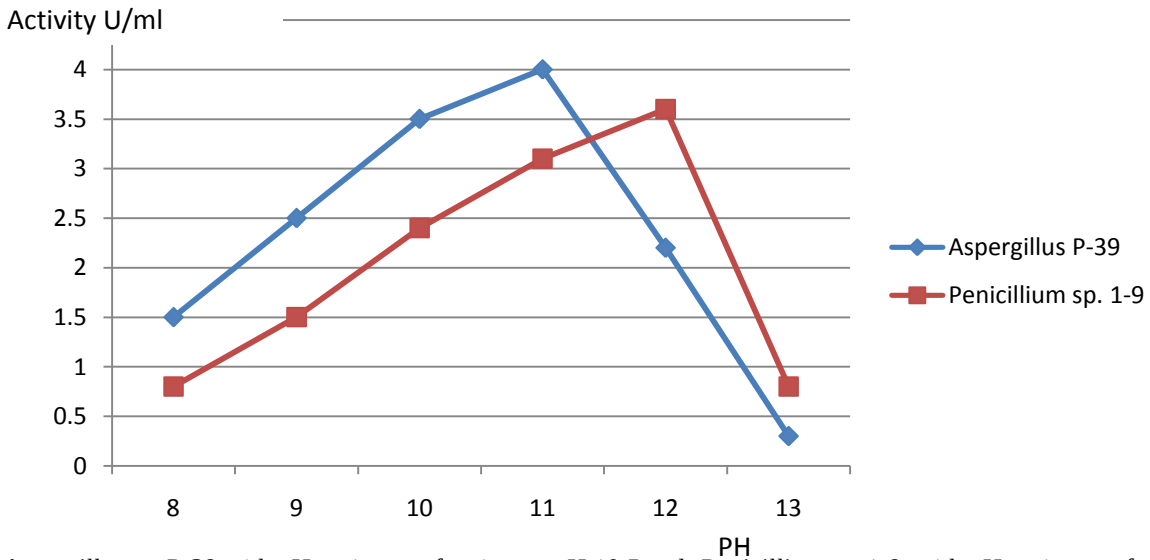
Fig. 2. Effect of temperature on the action of proteases



Strain -*Penicillium* sp. T 1-10; Mucor sp.T 44; protease activity optimal temperature is 70 ^{O}C, Penicillium sp. To 1-10 – 55 ^{O}C and Aspergillus sp. T 1-3 – 60 ^{O}C.

pH optimums of enzymes produced by these cultures were studied. Incubation area pH changed between 8.0 and 12.0 (0.05 M Na-phospate buffer)

Fig. 3. Proteases pH optimums of action



Aspergillus sp. P-39 with pH optimum of action at pH 10.5 and *Penicillium sp.* 1-9 with pH optimum of action at pH 11.5

Conclusion

Selection of microorganisms for last two, three decades has proved that stable forms of enzymes are more expedient to searche among the microorganisms possessing optimum growth at unusual conditions [17] In order to obtain stable enzymes of micromicetes, cultivation of producers under extreme conditions attracts peculiar interest. Not all enzymes, formed by extremophiles have increased stability in comparison with their mesophiles analogues. However, there are quite a few examples that some enzymes produced by fungi extremophiles, do not require additional stabilization [18].Large amounts of patents, original publications, reviews and monographs are devoted to the ways of application of hydrolytic enzymes. In 2007, "Springer" has published the reviews devoted to the manufacture and application of enzymes. Peculiar interest attracts the

material devoted to industrial enzymes [19,20.] in which the separate chapters are devoted to the application of enzymes in food processing and other branches of industry and in agriculture. Traditionally, proteases of fungal and bacterial origin have been long time used in different branches of food processing.

Collection of micellar fungi isolated from different ecological niches of South Caucasus has been created in Durmishidze Institute of Biochemistry and Biotechnology. As a result of screening among collection strains 39 producers of protease were selected with different degree of proteases activities (Table 1.). As a result of optimization of nutrient medium the activities of proteases produced by strains were increased by 22-50%. Technical preparations of proteases were obtained by precipitation with $(NH_4)_2SO_4$ (70g/100ml). In order to define temperature optimums for protease enzyme preparations, enzyme activity was measured between 20-80^0C with 5^0 intervals. In order to define pH optimums of enzyme activity, pH of incubation medium was changed from 2.0 to12.0, by pH 0.5 intervals. Optimum temperatures of action of protease preparations from three producers - *Mucor sp.*T-44, *Penicillium sp.*T 1-10 and *Aspergillus sp*. 1-3 were estableshed to be within the range 60-70°C *Mucor sp.*T-44 protease activity optimal tempetature is 70°C **.** *Penicillium sp.*T 1-10 -55 °C and *Aspergillus sp*. 1-3 - 60 °C (Fig. 1; 2.) Especially considerable are preparations from alkaliphile *Aspergillus sp* P-39 with pH optimum of action at pH 10.5 and *Penicillium sp.* 1-9 with pH optimum of action at pH 11.5 (Fig 3). Performed experiments revealed that selected preparations of proteases can be successfully used in different branches of industry.

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