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Extremophilic Actinomycetes, Distributed in Various Types of Soils of Georgia and their Protease Activity

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ABSTRACT. Protease activity of actinomycetes isolated from various soil types of Georgia has been studied. Protease activity strains have been identified among mesophilic and extremophilic actinomycetes. Some strains of mesophilic, alkaliphilic and halophilic actinomycetes are characterized by alkaline protease activity. Their pH optimum for enzyme activity is 10-11.

Strains of mesophilic actinomycetes: *Streptomyces glaucus* 71MD, *Streptomyces levoris* 114D, *Streptomyces coelicolor* 774 are of great interest. Their enzymatic activity at pH-11 corresponded 2.5unit/ml, 1.3unit/ml, 2.3unit/ml, respectively. Alkaliphilic actinomycetes strains *Streptomyces streptomycini* 204A and *Nocardia paloris* 206A with protease activity 0.85unit/ml and 1.1unit/ml respectively are also important strains. Optimum pH of enzyme activity is 10. Halophilic strain spp. 387H reveals maximum enzyme activity at pH-10 – 1.2unit/ml. *© 2010 Bull. Georg. Natl. Acad. Sci.*

Key words: *alkailprotease activity, mesophilic actinomycete, extremophilic actinomycete.*

Study of extremophilic actinomycetes and identification of their metabolic properties are most important tasks in biotechnology. The ability to produce a variety of proteolytic enzymes from terrestrial sources is a well-known phenomenon in actinomycetes [1].

Distribution of mesophilic actinomycetes, the producers of proteolitic enzymes in various types of soils of Georgia, has been studied. Pure cultures have been isolated. Some of them are of high extracellular activity, in particular, *Streptomyces fradiae* 110. It has been found that the enzymatic complexes isolated from *Streptomyces fradiae* 110 strain perform hydrolysis of elastine, casein, albumin, soybean proteins, food yeasts and wheat residues (from 8% to 96%) to free amino acids and low molecular peptides [2, 3].

The stability of enzymes produced by extremophiles much exceeds that of the ordinary microorganisms and enzymes of nonmicrobial origin. It is of interest to note that 70% of the total amount of enzymes are proteolytic enzymes and 25% of protease commercial preparations account for alkaline proteases. Among thermostable protease producers alkaliphilic actinomycetes - *Thermoactinomyces* sp., *Streptomyces* spp. have been found [4].

Proteases can be industrially produced in large amount and are economically important for the detergent, protein, brewing, meat, photographic, leather, and dairy industries. Proteases are common enzymes in plant and animal tissues, fungi and bacteria. Microorganisms are the preferred protease producers, as they grow rapidly and require little cultivation area, and can easily be subjected to genetic manipulation. The possible use of *Streptomycetes* for enzyme production has recently been investigated. Several proteases were obtained from streptomycetes. Their biochemical characterization, for example, of serine protease, produced by *Streptomyces*

pactum, metallo- and serine proteases from *Str. exfoliatus* and aminopeptidase from *Str. rimosus* has been carried out [5].

Materials and Methods. The goal of the present investigation was to isolate and characterize extremophilic streptomycete strains and define their proteolytic activity. Isolates of actinomycetes with high proteolytic activity were taxonomically identified.

Anson's method, modified by Petrova, was used to establish protease activity [6, 7]. The studied cultures were incubated by deep cultivation 72 h in order to establish protease activity. TSB was used for thermophiles [8], and soya flour containing medium, modified by the authors, was used for halophiles.

Anson's method, modified by Petrova and Vintsyunaite, was used to define protease activity: casein 2% solution was used as a substrate (2 g casein in 100 ml 0.05 M phosphate buffer, pH – 8.0). Standard curve was built according to tyrosin; pH change from 8 till 12 was conducted to establish optimum of activity of proteolytic enzymes. Substrate (casein) was dissolved in 0.05 M Na-phosphate buffers with different pH. Enzyme dilution was performed in corresponding pH buffers. Activity was studied by the above mentioned methods.

The culture was cultivated in liquid nutrient medium, on a shaker, 30^0C, at 180 rev/min, 120 hr. Nutrient medium, g/l: starch – 40, soya flour – 10, $(NH_4)_2SO_4$ – 0.65, K_2HPO_4 – 0.45, $CaCO_3$ – 3, $ZnSO_4$ – 0.2, $FeSO_4$ – 0.1, $MnCl_2$ – 0.1 was used.

Morphology of the isolated actinomycetes, their phases of development were studied by light microscope at ×200-300. The forms of spore surface were studied by electron microscope, at ×19000-23000. Growth ability of the culture was studied on different synthetic and organic nutrient media. Pridham's method was used to study the ability of carbon source uptake. 1% source of carbon, in particular, monosaccharides – glucose, fructose, galactose, arabinose, xylose, ramnose, alcohols – mannitol, sorbitol, inositol, dulcite, glycerol, disaccharides – saccharose, lactose, maltose, polysaccharides - starch, organic acids – sodium citrate, sodium lactate, and sodium succinate was added to nutrient medium [9]. Fedorov's nutrient medium was used to establish uptake of different sources of nitrogen. Nitrogen containing organic and inorganic compounds were used as nitrogen source.

The ability of actinomycetes to grow on hydrocarbons – crude oil, hexane, benzpyrene containing nutrient medium was investigated. The ability of actinomycetes to hydrocarbon absorption was defined according to growth intensity.

Antagonistic properties were studied by the agar block method [10]. As test-cultures the following strains were selected: *Staphilococcus aureus, Escherichia coli, pseudomonas aeroginosa, Azospirillum brassilense* G-3, *Mycobacterium phlei, Rodoccocus* spp., *Saccharomyces cerevisiae, Candida utilis, Rhizoctonia* spp., *Fusariom solani.*

Results and Discussion. We have studied the distribution of extremophilic actinomycetes in various types of soils of Georgia: solonets, solonchak, cinnamonic, meadow cinnamonic, cinnamonic calcareous, cinnamonic leached, raw humus calcareous, chernozems, leached chernozems, mountain meadow soddy, mountain forest meadow, mountain meadow soddy peat, mountain meadow chernozem like, brown forest podzolized, subtropical ortstein podzols, red soils, alluvial saturated.

197 pure cultures have been isolated from saline and solonchak soil samples of Eastern Georgia. According to salinity these soils are attributed to sulphate or chloride-sulphate types. High salinity is characteristic for soils of Sagarejo district (village Krasnogorka), where dry residue amounts 3.447-3.994% [11]. It has been established that the highest limit for NaCl concentration required for growth and development of halophilic actinomycetes is 10%. 6 genera: *Streptomyces* - 65%, *Nocardia* – 14%, *Streptosporangium* – 7%, *Saccharopolyspora* - 3%, *Micromonospora* – 6%, *Promicromonospora* – 5% have been revealed during the process of study [12].

The distribution of halophilic actinomycetes in various types of soils of Western Georgia: (Racha – Oni district and Ambrolauri district, Vani, Zugdidi districts, Poti district – Paliastomi, Maltakva) and Southern Georgia (Bakuriani, Akhaltsikhe, Akhalkalaki, Aspindza, Ninotsminda districts) has been investigated. 40 cultures of halophilic actinomycetes from yellow, raw humus calcareous, subtropical ortstein podzol soil samples of Western Georgia were isolated and 58 cultures from cinnamonic, chernozem, mountain meadow chernozem-like and brown forest podzolized soils of Southern Georgia were found. Totally 295 pure cultures of halophilic actinomycetes have been isolated.

Among halophilic actinomycetes 48 strains demonstrate protease activity. It varied from 0.03 to 1.52 unit/ml. Among the studied cultures protease activity was found in 48% of actinomycetes from the environs of Lake Kumisi, 11% - from Krasnogorka, 26% - from millary valley and 15% - Alazani valley. Highly active protease producers are strains: *Streptomyces rectiviolaceus* 173H, isolated from the environs of Lake Kumisi is distinguished for protease activity – accoun-

ting for 1.23 units/ml, *Nocardia* spp. 286H – 1.05 unit/ml, from soils of Alazani valley, *Streptomyces streptomycini* 295H – 0.9 unit/ml from Milary valley and *Streptomyces* spp. 387H – 1.2 unit/ml from subtropical podzolic soils of Zugdidi.

We have studied the dependence of these cultures on temperature. The optimum temperature is 28 - 30^{0}C. Moderate growth of *Streptomyces rectiviolaceus* 173H at low temperature, 3^{0}C, was found. The growth ability of strains in response to different pH of agar medium was studied. The study of *Streptomyces rectiviolaceus* 173H revealed that culture grew well at pH 7 to 12**.** On the basis of morphological properties it was found that the studied cultures mainly developed long or short straight sporiferous aerial mycelium. The cultures with straight sporiferous aerial hyphae have smooth spore surface.

Physiological and biochemical properties of the isolated actinomycetes have been studied. The strains differ by their ability to take up different sources of carbon and nitrogen. Strain *Streptomyces rectiviolaceus* 173H intensively takes up glucose, galactose, maltose, glycerol, starch from carbohydrate sources. Good uptake of KNO_3, $NaNO_3$, $Ca(NO_3)_2$, peptone, asparagine, lysine, leucine, b-alanine, aspartic acid from nitrogen sources has been observed.

It has been found that this strain obtains nitrate reductase and protease activities, is capable of milk peptonization and coagulation, gelatine dilution. It reveals ability to starch hydrolysis, and obtains no antagonistic properties gramnegative bacteria, mycobacteria and yeast. Intensive uptake of hydrocarbons: naphthalene, hexane has been observed [13].

185 pure cultures of alkaliphilic actinomycetes have been isolated from various types of soils of Georgia among them: 115 – from soils of Eastern Georgia, 39 – Western Georgia and 31 – Southern Georgia. The distribution of alkaliphilic actinomycetes in raw humus calcareous (37%) and cinnamonic calcareous (25%) soils has been established.

As a result of alkaliphilic actinomycetes identification 3 genera have been revealed: *Streptomyces, Nocardia, Streptosporangium.* Most of the investigated strains are attributed to *Streptomyces* genera. Among the investigated strains 70% are *Streptomyces,* 17.5% – *Streptosporangium,* 12.5% - *Nocardia* [14].

In alkaliphilic actinomycetes protease activity fluctuates within 0.3-1.2 unit/ml limits. Protease activity was found in strains isolated from Kaspi district soil samples– 14%, Dedoplistskaro – 36%, Dmanisi district – 31%, Stepantsminda – 73%, Sagarejo district – 48%. Two strains - *Streptomyces globisporolactis* 203A (1.2unit/ml), *Streptomyces streptomycini* 204A (0.85 unit/ml) from Stepantsminda, *Nocardia paloris* 206A (1.1 unit/ml) from Sagarejo district are of particular interest.

The biological properties of alkaliphilic actinomycetes have been studied.

Streptomyces globisporolactis 203A – aerial mycelium – straw-colored, colonies and nutrient medium – colorless, long, straight weaved hyphae, with good fragmentation, smooth surface spores.

Of carbohydrate sources good uptake of glucose, galactose, xylose, maltose, mannitol, glycerol, sodium lactate, starch was observed. Of nitrogen sources it takes up KNO_3, $Ca(NO_3)_2$, peptone, asparagine, lysine, leucine, b-alanine, arginine, valine.

It forms H_2S, obtains nitrate reductase and protease activities. It is capable of milk peptonization and melanoid pigment production. It is capable of starch hydrolysis and obtains no antagonistic properties. Optimal conditions of growth pH – 7-11, 28-30^{0}C. Weak uptake of hydrocarbons: naphthalene, hexane, benzene, crude oil has been observed.

Nocardia paloris 206A - aerial mycelium grey, greyish, colonies and nutrient medium colorless, with long, straight hyphae, fragmented, smooth surface spores.

The strain intensively takes up carbohydrates: glucose, galactose, saccharose, lactose, maltose, glycerol, sodium lactate, starch, cellulose and of nitrogen sources KNO_3, $Ca(NO_3)_2$, peptone, asparagine, lysine, leucine, b-alanine, arginine, valine.

It weakly transforms hydrocarbons, and intensively grows on 0.2%, 0.5% and 1% containing 2.4-D nutrient medium, obtains no ability to starch hydrolysis, obtains protease activity. pH – 7-11. It is capable of milk peptonization, gelatine dilution and melanoid pigment and H_2S production. No milk coagulation has been recorded. Intensive suppression of *Staphilococcus aureus, Saccharomyces cerevisiae* and no action against gramnegative bacteria, mycobacteria and fungi has been observed.

Thermophilic actinomycetes from various types of soils of Georgia, collected in different seasons of the year have been used for investigation. In all 97 pure cultures of thermophilic actinomycetes of *Thermoactinomyces* genera have been isolated; their growth optimum is 50-55^{0}C, and they do not grow at 28^{0}C. Most of the pure cultures of thermophilic actinomycetes have been isolated from Tsalka chernozems and Borjomi district mountain meadow soddy, mountain meadow chernozem-like, mountain meadow soddy peat

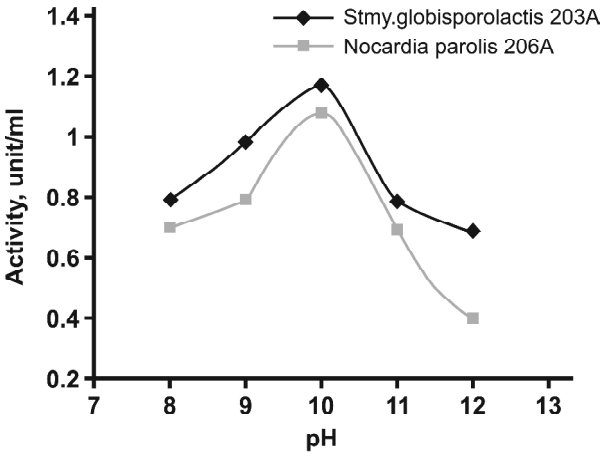

Fig. 1. Protease activities of alkaliphilic actinomycetes.

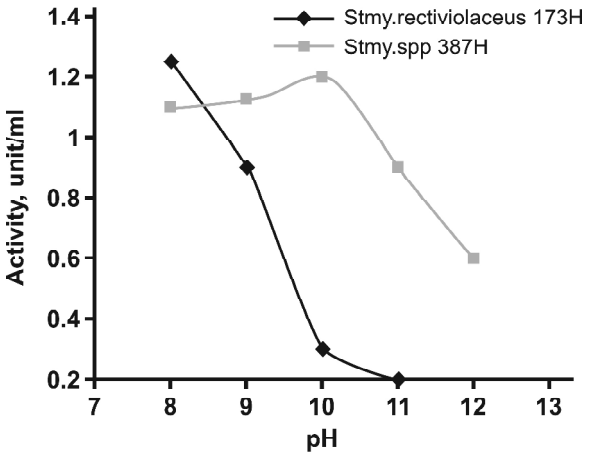

Fig. 2. Protease activities of halophilic actinomycetes.

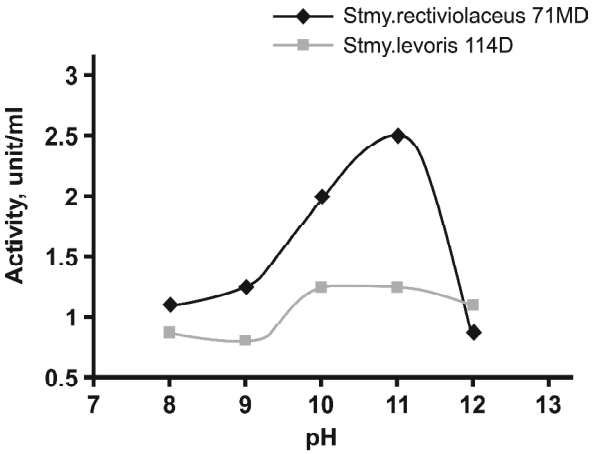

Fig. 3. Protease activities of mesophilic actinomycetes.

soil samples. To determine protease activity thermophilic actinomycete cultures were cultivated in liquid nutrient medium – TSB, in thermostat, at 55^0C, 72 hr. Enzyme activity was stated at 40^0 and 50^0C. It should be underlined that enzyme activity of some strains increased at 50^0C. Enzyme activity of different strains fluctuates within 0.2-0.57 unit/ml limits. Protease activity was found in those strains which are characterized by gelatin thinning ability.

Factors such as temperature, pH, sodium chloride concentration, different carbon and nitrogen sources and aminoacids which influence the secretion of protease enzyme by actinomycetes were optimized for maximum production [1].

Among strains with protease activity 15 strains, capable of alkaline protease production have been revealed. Their pH optimum of enzyme activity is within 10-11 limits. Some strains revealed enzyme activity at pH – 12 (Fig. 1-3). Alkaliphilic actinomycetes from soils of Stepantsminda and Sagarejo districts are worth mentioning. Enzyme activity of *Streptomyces globisporus* 203A, isolated from mountain-meadow soddy soils of Stepantsminda district at pH – 10 is equal to 1.2 unit/ml (Fig. 1). Activity of the strain decreases at pH – 12 and accounts for 0.7 unit/ml. Strain *Streptomyces streptomycini* 204A and *Nocardia paloris* 206A isolated from brown calcareous soils of Sagarejo district are of great interest. Their protease activity corresponds to 0.85unit/ml and 1.1unit/ml respectively. Optimum pH of enzymatic activity is 10 and activity is retained at pH – 11-12. Maximum enzyme activity of the strain *Streptomyces* spp. 387H has been reveaved at pH –10 – 1.2unit/ml (Fig. 2).

Producers of alkaline proteases have been also found among mesophilic actinomycetes: *Streptomyces glaucus* 71MD, *Streptomyces levoris* 114D, *Streptomyces coelicolor* 774. Their enzymatic activity at pH-11 corresponded to 2.5unit/ml, 1.3unit/ml, 2.3unit/ml, respectively (Fig. 3).

As a result of the investigations carried out it has been established that protease producers are observed among mesophilic, as well as halophilic and alkaliphilic actinomycetes.

მიკრობიოლოგია

საქართველოს სხვადასხვა ნიადაგში გავრცელებული ექსტრემოფილური აქტინომიცეტები და მათი პროტეაზული აქტივობა

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ნაშრომში გამოკვლეულია საქართველოს სხვადასხვა ტიპის ნიადაგიდან გამოყოფილი აქტინომიცეტების პროტეაზული აქტივობა. პროტეაზული აქტივობის მქონე შტამები გამოვლენილ იქნა მეზოფილურ და ექსტრემოფილურ აქტინომიცეტებში. მათ შორის მეზოფილური, ალკალიფილური და ჰალოფილური აქტინომიცეტების ზოგიერთი შტამი ხასიათდება ტუტე პროტეაზული აქტივობით. მათი ფერმენტული აქტივობის pH ოპტიმუმი 10-11-ის ფარგლებშია.

აღსანიშნავია მეზოფილური აქტინომიცეტების შტამები: *Streptomyces glaucus* 71MD, *Streptomyces levoris* 114D, *Streptomyces coelicolor* 774. მათი ფერმენტული აქტივობები pH-11-ის პირობებში შესაბამისად არის 2.5 ერთ/მლ, 1.3 ერთ/მლ, 2.3 ერთ/მლ; ალკალიფილური აქტინომიცეტების შტამები - *Streptomyces streptomycini* 204A და *Nocardia paloris* 206A, რომელთა პროტეაზული აქტივობები შესაბამისად შეადგენს 0.85ერთ/მლ და 1.1ერთ/მლ-ს. ფერმენტული აქტივობის pH ოპტიმუმი არის 10. ჰალოფილური შტამი *Streptomyces* spp. 387H მაქსიმალურ ფერმენტულ აქტიურობას ავლენს pH 10-ის პირობებში - 1.2ერთ/მლ.

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