ENZYMATIC ACTIVITY OF NITROGEN-FIXING BACTERIA ISOLATED FROM ARMENIAN SALINE SOILS

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CZU:579.26:631.46 https://doi.org/10.52757/imb22.38

Summary

Nitrogen-fixing bacteria with phytostimulating potential have great interest in the modern world. The aim of this work was to study the enzymatic activity of nitrogen-fixing strains *Agrobacterium* sp. M-1 and *Agrobacterium* sp. Y-2. Studies have shown that these strains are capable to produce proteases, lipases, cellulases, amylases and ureases, enzymes that play a vital role in maintaining soil health. In addition, these bacteria have several significant characteristics: salt tolerance, pH stability, maintaining of viability at low and high temperatures. Therefore, the above strains have every chance to become the basis for the creation of a multifunctional biofertilizer in the future.

Keywords: soil enzymes, biological catabolism, microbes, soil health, nirogen-fixing bacteria

Introduction

Soil is a medium for more than 100 enzymes that play a fundamental role in supporting soil ecology and health. They are direct agents of the biological catabolism of the organic and mineral components of the soil and with their help the decomposition of organic residues and the cycle of nutrients occur in the nature. During soil deterioration the change of enzymes occurs much sooner than of other parameters in the soil. That's why they are the best indicators of soil health and by evaluation of their activities it is possible to determine whether soil processes are proceeding satisfactorily [1].

In a number of potential bacterial enzymes that play an important role in maintaining soil health and in the transformation of different nutrients for plants, some of the important ones are protease, lipase, cellulase, amylase and urease. High productivity of crops is associated with the activity of these enzymes, since they convert plant and animal waste into humus, which is then completely decomposed into free nutrients consumed by plants [2].

Proteases are degradative enzymes, catalyzes proteolysis - breaking down of proteins into smaller polypeptides or single amino acids. They are present in all forms of life, such as animals, plants, and microbes. Proteases are regulators of physiological processes, controlling the activation, synthesis and turnover of proteins. Microbial proteases are the most used enzymes worldwide and account for two thirds of commercial proteases. They are preferred over plant and animal proteases due to having all the characteristics required for industrial applications: high productivity, less consumption of time, less requirement of space, high genetic manipulation and cost effectiveness [3-4].

Lipases are family of versatile enzymes that catalyze triglycerides into free fatty acids and glycerol. They can be obtained from several sources: animal, plants, and microbes. Lipase has a wide spectrum of activity and is involved in many reactions [5]. Lipase activity is one of the primary values for all living organisms, since it balances physiological processes of digestion and absorption, as well as the metabolism of fats and lipoproteins. Thereby, lipases are multi-purpose biological catalysts and are used in several industries such as biodiesel, food, textiles, pharmaceuticals, medicine, etc., due to their substrate specificity and ability to catalyze reactions at extreme pH, temperature and presence of metal ions [6].

Cellulase is an enzyme from the class of hydrolases that breaks down the cellulose molecule into monosaccharides, shorter polysaccharides and oligosaccharides. Cellulose is a widespread biomass in the nature, since it is the main component of cell walls of plants. Abundant availability of cellulose makes it an attractive raw material for producing many industrially products, cause can be converted to glucose which is a multiutility product. It can be done by the cellulolysis - the process of cellulose hydrolysis, which is basically the biological process controlled by the enzymes of cellulase system [7]. Cellulase enzyme system comprises three classes of soluble extracellular enzymes and only the synergy of them makes the complete cellulose hydrolysis to glucose. Many microbes, mostly bacteria and fungi have a cellulosic activity, but potential of bacteria is better due to their high rate of growth [8].

Amylases are starch-degrading enzymes, which occupy a quarter of all enzymes used in the global industry. Amylases are found in microorganisms, plants and animals, but in industrial processes such as food and pharmaceutical industries have basically used microorganism amylases, mainly bacteria and fungi by their higher stability and easy manipulating. Some of the major industrial enzymes from group of amylases are α-amylases preferred due to their ability act at high temperatures and alkaline pH. Function of α-amylases is hydrolyzed of α-1,4-glycosidic linkages of the starch leading to the formation of dextrins. Starch is a complex carbohydrate that exists in many vegetables and fruits. Plants create these polymers for storing of glucose, creating during process of photosynthesis [9-10].

Urease is a widespread in nature nickel-containing metalloenzyme, catalyses the hydrolysis of urea to ammonia and carbamate, and thus generates the preferred nitrogen source of many organisms. Ureases are found in numerous bacteria, fungi, algae, plants, and some invertebrates. It is a predominant enzyme among the soil N cycle enzymes. Urease activity is a biological indicator of the soil. It allows to management the amount of urea fertilizer added to the soil, because as a result of the hydrolysis of urea-containing fertilizers by bacterial ureases, an increase in soil pH occurs. Subsequently, excessive addition of urea fertilizer to the soil can be prevented [11-13].

Some of the representatives of microbes with enzymatic activity are nitrogen-fixing bacteria, such as *Rhizobium*/*Agrobacterium* group, genera *Azotobacter* and *Pseudomonas*, etc. They have a good effect on the growth and development of the crops, leading to an increase in the yield. They enhance biological nitrogen fixation, help in the synthesis of biologically active substances, make certain nutrients available to plants such as C, N, P, and also protect plants from phytopathogens because they are good antagonists [19, 25, 27].

Nitrogen is one of the most abundant elements in surface and atmosphere of Earth; moreover, it's one of the essential macronutrients for plant growth. Unfortunately, molecular nitrogen is not available for uptake by plants. Plants absorb nitogen in the form of ammonium salts, which are formed in the soil due to nitrogen-fixing bacteria. The latter convert molecular nitrogen fixed from the atmosphere into ammonia, which transfomed into ammonium salts in the soil. That's why bacteria supporting nitrogen fixation and plant growth are undoubtedly the most the vital resources for increasing productivity in agriculture [14].

The aim of this work was the study of enzymatic activities of protease, lipase, cellulose, amylase and urease in nitrogen-fixing bacteria isolated from saline soils of Armenia.

Materials and methods

Strains: The bacteria used in the experiments were *Agrobacterium* sp. strain M-1 (Genbank accession number: MN721294) and *Agrobacterium* sp. strain Y-2 (Genbank accession number: MN717167), representatives of *Rhizobium*/*Agrobacterium* group, previously isolated by us from saline soils of the villages Mrgashat and Yeghegnut of Republic of Armenia, respectively [15].

Growth medium: Burk's Agar was used as a nutrient medium with the following composition: 20.0 g/l sucrose, 0.640 g/l K2HPO4, 0.160 g/l KH2PO4, 0.20 g/l MgSO4 7H2O, 0.20 g/l NaCl, 0.050 g/l CaSO4 2H2O, 0.50 g/l Na2MoO4 · 2H2O, 3 g/l FeSO4 7H2O, 15.0 g/l agar; pH 7.3 [16].

To determine the enzymatic activity of the studied nitrogen-fixing bacteria, special mediums were prepared: Skim Milk Agar for proteolytic activity, Burk's Agar with Tween 80 for lipolytic activity, Congo-Red Agar for cellulolytic activity, Starch Agar for amylolytic activity and Urea Broth for ureolytic activity.

Skim Milk Agar – 5.0 g/l pancreatic digest of casein, 2.50 g/l yeast extract, 1.0 g/l glucose, 70.0 g/l skimed milk powder, 15.0 g/l agar; pH 7.0 [17].

Burk's Agar with Tween 80 – composition of the Burk's medium with the addition of 2% Tween 80 (v/v); pH 7.3 [16].

Congo-Red Agar – KH_2PO_4 – 0.5, $MgSO_4$ – 0.25, cellulose – 2.0, Congo-Red – 0.2, gelatin – 2.0, agar – 15.0; pH 6.8–7.2 [8].

Starch Agar – 3.0 g/l beef extract, 10.0 g/l soluble starch, agar – 12.0; pH 7.5 [18].

Urea Broth – 9.50 g/l K2HPO4, 9.10 g/l KH2PO4, 20.0 g/l urea, 0.10 g/l yeast extract, 0.010 g/l phenol red; pH 6.8 [19].

Determination of enzymatic activity: In order to obtain fresh cultures for use in the experiments, the strains were each time pre-seeded on Burk's Agar and incubated for 2 days at 30 °C. Bacterial

suspensions were prepared in physiological saline solution (0.96%, w/v) with the 10^7–10^8 CFU/ml end concentration.

Proteolytic activity: Protease production was determined using Skim Milk Agar medium. 0.2 ml of bacterial suspension was added into punch holes (5 mm diameter) in the agar and plates were incubated at 30 °C during 3 days. Formation of clear zone around the holes was considered as availability of proteolitic activity [17].

Lipolytic activity: Presence of lipase enzymes was determined by Burk's medium with Tween. Bacterial suspension was added into punch holes and Petri dishes were incubated at 30 °C during 5 days. The presence of a clear zone around the bacterial inoculum was indicated as the degradation of the Tween [20].

Cellulolytic activity: Cellulose degradation was study using Congo-Red Agar. Suspension was transferred into holes and was growth for 7 days at 30 °C. Manifestation of Congo red discoloration zone was taken as positive result [7].

Above three activities were measured by proteolytic index (PI), lipolytic index (LI) and cellulolytic index (CI):

$$PI\ or\ LI\ or\ CI = \frac{diameter\ of\ zone - diameter\ of\ punch\ hole}{diameter\ of\ punch\ hole}.$$

Amylolytic activity: Presence of amylases was by Starch Agar medium. A fresh colony of bacteria was streaked on the surface of the agar and was incubated for 3-5 days at 30 °C. Then the surface of the agar was flooded with Gram's iodine solution (3 ml). A clear zone surrounding the bacterial growth confirmed the hydrolysis of starch [21].

Ureolytic activity: Activity of ureases was investigated by using Urea broth. 0.1 ml of bacterial suspension was added into test tubes with broth (5 ml) and incubated at 30 °C for 4 days. The appearance of a deep pink color indicated a positive result [19].

Statistical analysis: Data analysis was carried out ANOVA by Dunnett's test ($p<0.05$) using Minitab 17.1 statistical program. All the experiments were conducted at least for 3 times in triplicates.

Results and discussions

The role of nitrogen-fixing bacteria in agriculture is of great interest due to their phytostimulating potential, bioregulatory properties and large number in the rhizosphere. Moreover, their enzymatic activities are also important for soil health and ecology, as evidenced by various literary data [20-22].

Enzymes are direct agents of the biological catabolism of the organic and mineral components of the soil. High productivity of crops is associated with the activity of these enzymes, since they convert plant and animal waste into free nutrients consumed by plants. Enzymes are present in all forms of life, such as animals, plants, microorganisms, etc. [13]. In worldwid the most used are microbial enzymes which are preferred due to their high productivity, less consumption of time and space, high genetic manipulation and cost effectiveness. In a number of potential bacterial enzymes that play an important role in soil health and in the transformation of different nutrients for plants, some of the importants are protease, lipase, cellulase, amylase and urease: enzymes that hydrolyze proteins, fatty acids, cellulose, starch and urea, respectively [2]. In addition, it is known that secretion of lytic enzymes (lipase, cellulase and protease) by microbes can contribute the inhibition of plant pathogens' growth [20].

In order for such potential bacteria in the composition of biofertilizers to be useful for plants, they must at first successfully take root in the soil, and then be able not only to survive, but also to maintain their vital activity, which is not always possible due to various harsh environmental conditions. That's why, in this work were study the nitrogen-fixing bacteria *Agrobacterium* sp. strain M-1 and *Agrobacterium* sp. strain Y-2 with Genbank accession numbers MN721294 and MN717167 respectively, which were previously isolated by us from saline lands of Armenia (with 2.1% average salt value, w/v) [22]. In addition of salt tolerance, these bacteria are also resistant to various pH values (in the range from 6 to 10) and are able to maintain their viability in conditions from low to high temperatures (in the range from -20 °C to +55 °C) [15].

Screening media Skim Milk Agar, Burk's Agar with Tween 80, and Congo-Red Agar were used to determine proteolytic, lipolytic, and cellulolytic activity, respectively. With a positive result, a clear zone was formed around the hole punch, the diameter of which was measured and, on its basis, the enzyme index

was determined. The results of enzymatic activities study indicated that both strain of *Agrobacterium* (M-1 and Y-2) were positive in the lipase test and cellulase test, but protease test was shown positive result only in strain Y-2 (Table 1).

Table 1. Proteolytic, lipolytic and cellulolytic activities of nitrogen-fixing bacteria

№	Nitrogen-fixing bacteria	Enzymatic activity					
		Protease		Lipase		Cellulase	
		Zone (mm)	PI	Zone (mm)	LI	Zone (mm)	CI
1	*Agrobacterium* sp. strain M-1	60	5.0	65	5.5	52	4.2
2	*Agrobacterium* sp. strain Y-2	58	4.8	42	3.2	45	3.5

There are many data in the literature about the enzymatic activities of nitrogen-fixing bacteria. In one were shown that almost all study *Pseudomonas* strains have protease activity, in contrast to lipases and cellulases, which were found only in *P. chlororaphis* TSAU13 [20]. A similar result was observed by Jha et al. where fluorescent strains of *Pseudomonas* sp. were shown proteolytic activity, but only one of them has cellulases [17]. Among the representatives of *Rhizobium*, the presence of proteolytic activity is most common, for example proteases were finding in nine isolates of *Rhizobium* sp. from Indonesian soils [23] as well as in *Rhizobium* sp. strain R-986 and *Bradyrhizobium* sp. strain R-993 isolated from the soils of the Central Amazonian floodplain [24]. In addition, De Oliveira et al. discovered that maximal protease activities were exhibited when the cell growth reached the stationary phase [24]. In case of genus *Azotobacter* there are many studyes showing absence of cellulolytic activities and presence of protease activity [25-26]. Alsalim showed *Azotobacter chroococcum*'s ability to produce proteases and lipases [27].

In the case of determination of amylolytic activity, after incubation, the surface of the starch agar was covered with a solution of Gram's iodine, which reacts with starch and stains the agar in a dark blue color. The appearance of a light zone around the bacterial growth indicates the hydrolysis of starch by the bacteria due to the production of amylases [28]. Results were shown that both *Agrobacterium* sp. strain M-1 and *Agrobacterium* sp. strain Y-2 have amylolytic activity (Fig. 1a).

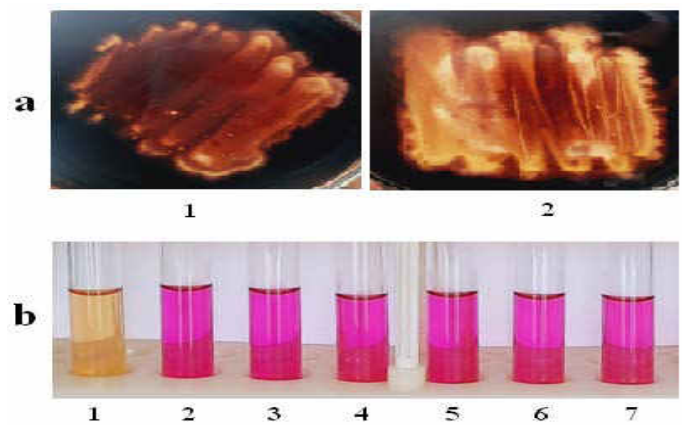

Figure 1. Amylolytic and ureolytic activities of of nitrogen-fixing bacteria

a) amylolitic activity - *Agrobacterium* sp. strain M-1 (a1) and *Agrobacterium* sp. strain Y-2 (a2) with positive result: the appearance of a light zone around the bacterial growth indicates the hydrolysis of starch

b) ureolytic activitiy – Control of Urea Broth (b1), *Agrobacterium* sp. strain M-1 (b2-b4) and *Agrobacterium* sp. strain Y-2 (b5-b7) with positive result: the pink color will develop due to the hydrolysis of urea and the formation of ammonia, leading to a change in the pH of the medium from neutral to alkaline

Study of ureolytic activity was done by Urea broth medium. Positive result was considered when the medium went from light orange to pink. The pink color was formed due to a change in pH from neutral to alkaline, which occurs due to the formation of ammonia in the medium in consequence to the hydrolysis

of urea by the urease enzymes [29]. The study of ureolytic activity showed that in all the studied samples the color of the medium became pink, i.e. they have a positive result (Fig. 1b).

There are data in the literature about starch hydrolysis by different nitorgen-fixing bacteria, e.g., by *Azotobacter chroococcum* [30] and by all studied isolates of *Pseudomonas* except Rh01 and Rh3 by Jha et al. [17], but *Rhizobium* isolates, located in symbiotic relationship with Groundnut, gave negative results for starch hydrolysis [21]. In case of urease activity positive result is meet in upland cotton-associated bacterial strains *Azotobacter chroococcum* AC1 and AC10 [25] and in all isolates of Jha et al. [17].

As seen from the literature data, the picture of enzymatic activities is very diverse and depends from various factors, for example the genus and species of bacteria, the environment conditions and so on.

Based on the research findings, it can be assumed that the use of *Agrobacterium* sp. strain M-1 and *Agrobacterium* sp. strain Y-2 with a variety of enzymatic activities can provide the soil with the enzymes necessary for the normal course of global carbon and nutrient cycles.

Conclusions

The work resulted in the detection of the variety of enzymatic activities in bacteria Agrobacterium sp. strain M-1 and Agrobacterium sp. strain Y-2. In addition, previous studies have shown their salt tolerance (under until 2.1% salts existence in environment), pH stability (in the range 6-10) and maintain of their viability in low to high temperatures (in the range from -20 °C to +55 °C). Based on these positive qualities, Agrobacterium sp. strain M-1 and Agrobacterium sp. strain Y-2 strain have every prospect of becoming the basis for the creation of a multifunctional biofertilizer.

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