

# Resistance of various soil geosystem bacteria to enzymatic lysis at different pH

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**Abstract.** Bacteria appear to be an inevitable element of soil geochemistry because they participate actively in chemical transformations of soil media components. One of the factors determining the composition of bacterial population in soil is resistance of bacteria to enzymes which hydrolyze cell walls. In the following research, resistance of bacteria (*Priestia megaterium*, *Micrococcus luteus* and *Escherichia coli*) to lysozyme in the pH range appropriate for soils is investigated. All the three species are proved to be most sensitive to lysozyme in slightly alkaline conditions (*P. megaterium* is least resistant at pH 8.0, *M. luteus* at pH 8.3, *E. coli* at pH 8.5), and resistance of all the three microorganisms in the range of pH values of 6.0—7.0 changes insignificantly. A possible factor defining the pH optimum for lysozyme activity in relation to these bacteria is the structure of negatively charged components of their cell walls and outer membranes.

## 1 Introduction

The bacterial population of soils performs a number of functions that are essential for the formation of the ecosystem: bacteria enrich the soil with nitrogen by means of atmospheric nitrogen fixation, solubilize components of underlying minerals (especially phosphorus), degrade complex organic compounds into simpler ones (i. e. function as decomposers), secrete various enzymes which catalyze necessary reactions for maintaining the carbon and nitrogen cycle in the ecosystem, and pH values of soil environments depend on bacteria activity [1].

Bacterial population of soil somehow deals with necessity of resisting bacteriolytic enzymes, secreted by other microorganisms as well as entering the soil from other organisms.

During their whole life cycle, bacteria undergo activity of various bacteriolytic enzymes which hydrolyze their cell walls. Various organisms, including bacteria itself, can produce bacteriolytic enzymes. The most widely known example of bacteriolytic enzymes is a family of lysozymes, which are hydrolases destructing polysaccharide chains of cell walls [2]. Lysozymes are produced by most animal species, including almost all vertebrates. Excluding enzymes of this family which are designed for digestion, lysozymes are most active at slightly alkaline pH values [3]. Another type of bacteriolytic enzymes is

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bacteriophage enzymes which are applied for the virus (bacteriophage) DNA to enter a host bacterial cell and for the newly synthesized phage units to exit the cell [4—6]. For phage enzymes, as well as for animal lysozymes, the pH optimum of the catalytic activity of bacterial lysis is characteristic in the slightly alkaline pH region [7]. An enormous variety of bacteriolytic enzymes with various structure, specificity and physicochemical properties is produced by the bacteria themselves [8—9]. Among the bacteriolytic hydrolases expressed by bacteria, we can distinguish autolysins (enzymes intended for the producer's own purposes, for example, to reorganize the cell wall during division or sporulation [10]), and enzymes released into the external environment that are applied by bacteria to fight other types of bacteria.

To investigate the resistance of microorganisms to enzymatic lysis, three species of bacteria were chosen for this research, which differ morphologically and in habitat conditions, but all the three can influence geochemical properties of soils.

*Priestia megaterium* (known as *Bacillus megaterium* until 2021), also called hay bacillus or grass bacillus, is a facultative aerobic gram-positive spore-forming bacterium, mostly populating soil and groundwater in nature [11]. From an ecological point of view, this is a species with extremely diverse metabolic capabilities; for instance, some strains of *P. megaterium* are known to fix atmospheric nitrogen [12—13]. This species also transforms phosphorus contained in

phosphate minerals into a soluble state [14] and can be applied for restoration of soils contaminated with heavy metals [15—17]. This bacterium is no less known and important as a probiotic in animal farming, including nutrition of invertebrates, fish, poultry, swine, and various cattle [18—22]. Application of *P. megaterium* in biotechnology is also very diverse: it is a producer of many enzymes (both recombinant and natural for the species) [23—25], vitamins [26], biosurfactants and polyhydroxyalkanoates. The diversity of carbon sources for *P. megaterium* is enormous [27]. In terms of temperature optimum, most strains of this species used in industry are mesophilic, but the range of temperatures suitable for *P. megaterium* to proliferate is from 3 to 45°C [28—29]. Such versatility of *P. megaterium* allows us to consider it as a promising microorganism in almost any sector of the economy that requires the use of bacteria. The ecology of the *Priestia* family is mostly similar to the ecology of some strains of *P. megaterium*, the species most diverse ecologically among them.

Gram-positive cocci of the genus *Micrococcus*, including *Micrococcus luteus*, which is very widespread as a model microorganism in biochemistry, are typical inhabitants of the skin of humans and some animals [30]. Pathogenic strains are rare for bacteria of this family. Besides symbiotic lifestyle, micrococci can live in soils, fresh and sea water, but the species *M. luteus* is never found in salt waters [31—32]. *Micrococcus luteus* strain ATCC 4698 and *Priestia megaterium* strain ATCC 14581 are included in the set of standard microorganisms when assessing the number of bacteria in agricultural soil using fast flow cytometry [33].

Typical habitats for the mesophilic gram-negative *Escherichia coli* are intestines and other internal organs of warm-blooded vertebrates, as well as soil and natural fresh waters, where bacteria enter with animal excretions [34—37]. Pathogenic strains of *E. coli* are quite widespread as causative agents of intestinal and other infections [38]. The content of some widespread species of bacteria (including *E. coli*) in natural environments is one of the indicators of the ecological state for these environments. For instance, exceeding concentration of *E. coli* cells (independently of strains and pathogenicity) is often a marker of soil contamination with waste products, since *Salmonella* and *Shigella* bacteria, which cause life-threatening infections, enter the soil from the same source [39—40]. There are standards for the content of *E. coli* in natural environments in several countries [41].

In several geographic areas, it has been shown that certain strains of *E. coli* may have the ability to colonize and adapt to soil conditions, which means that such populations are part of the natural soil microflora, potentially influencing the geosystem of nearby fields and reservoirs [42–43], including the prospect of using environmentally harmful substances in biodegradation processes [44]. The stability of *E. coli* activity depends on soil moisture: in hydromorphic soils, after entering from wastewater, *E. coli* does not disappear from the soil surface after more than 3 months and can be also found in deep soil layers of 30–40 cm [45]. The environmentally persistent *E. coli* strain isolated from the soil, compared to the reference strain K-12, exhibits the same viability at a temperature of 37°C, but is more adapted to the use of a wide range of nutrient sources at a temperature of 15°C [46].

Strains resistant to lysozyme are present among all three of bacterial species considered. For *E. coli*, the typical modifications of mureine (cell wall polymer) which produce lysozyme resistance are acylation at oxygen atoms or deacylation at nitrogen atoms of some units of N-acetylmuramic acid [47–48]. In addition, *E. coli* produces the periplasmic lysozyme inhibitor [49]. Acetylation at the oxygen atoms of the monosaccharide units of N-acetylmuramic acid is also present in lysozyme-resistant strains of *M. luteus* [50]. Peculiar mechanisms for *P. megaterium* are cross-linking of peptide bridges [51], and, to a lesser extent, O-acetylation of N-acetylmuramic acid and the presence of alanine residues in teichoic acids [52, 53].

Thus, the purpose of this research was to study the resistance to enzymatic lysis of bacteria that affect the geochemical parameters of soils (*P. megaterium*, *M. luteus*, *E. coli*) at different pH values in the range typical for different soils. Chicken egg lysozyme was used as a model enzyme.

## 2 Materials and methods

Reagents applied are: hen egg white lysozyme, Tris, MES (Amresco, USA), HCl (Komponent-Reaktiv, Russia), bactotryptone (BD Difco, USA), NaCl, NaOH (Panreac, Spain), glucose (RokettFrer, France), yeast extract (Biospringer, France), lyophilized cells of *Micrococcus luteus* ATCC4698 (Sigma-Aldrich, USA). *Priestia megaterium* strain ATCC 14581 (B-9869) and *Escherichia coli* strain K-12 (B-3254) are received from All-Russian collection of industrial microorganisms (National Research Centre "Kurchatov Institute").

Equipment applied in the research includes UV-1800 dual beam spectrophotometer (Shimadzu, Japan), LT-105a thermostat (LOIP, Russia), 5804R centrifuge (Eppendorf, Germany), MiniSpin centrifuge (Eppendorf, Germany), Explorer Pro EP114C analytical scale (OHAUS, Switzerland), Orion 120 pH-meter (Thermo, USA), ESLK-01.7 electrode (Akvilon, Russia), New Brunswick Innova 44 incubator shaker (Eppendorf, Germany).

### *Priestia megaterium* cultivation

*Priestia megaterium* cell culture was grown in LB nutrient medium, pH 7.6 [54] by two stages. At the first stage, cultivation was performed in 2 test-tubes with 10 mL of medium, with aeration, at 30°C, shaker speed 120 rpm, during 14–16 hours. The second stage of cultivation was carried out in 100 mL conical flasks, into which 1 mL of the *Priestia megaterium* culture obtained at the first stage was input. The second stage was also performed with aeration, at 30°C, shaker speed 120 rpm. Every 30 minutes, optical absorbance of the cell suspension at 650 nm was measured; cultivation was finished when this parameter reached 1.0–1.2.

Centrifugation of the cell culture obtained at the second stage was carried out in 50 ml tubes at 5804R centrifuge (Eppendorf, Germany), with F-34-6-38 rotor, during 30 min, at 6000 rpm (4811g), at the temperature of 4–5°C. The cell sediment was resuspended in 10

mL of 0.02 M Tris-HCl buffer solution, pH 8.0, with addition of 55 mM NaCl. 1.5 mL aliquots were frozen at  $-70^{\circ}\text{C}$ .

#### *Escherichia coli* and *Micrococcus luteus* cultivation

Bacteria were grown according to standard methods in liquid LB nutrient medium, pH 7.6 [55]. Suspension of cells (with the optical absorbance of 1.0 at 650 nm), prepared in 0.01 M Tris-MES buffer solution, pH 7.5, was frozen with liquid nitrogen by portions of 0.5 mL.

#### Preparation of bacterial cells for the experiment

Immediately before the experiment, aliquots of the cell suspension were defrosted and then centrifuged at 6000 rpm (2073 g) for 7 min in a MiniSpin centrifuge (Eppendorf, Germany). The cell sediment was suspended in the buffer solution applied for the following experiment: centrifugation and re-suspension were conducted twice. The resulting cell preparation was used within 1-1.5 hours after defrosting.

#### Measuring the activity of lysozyme towards bacterial cells

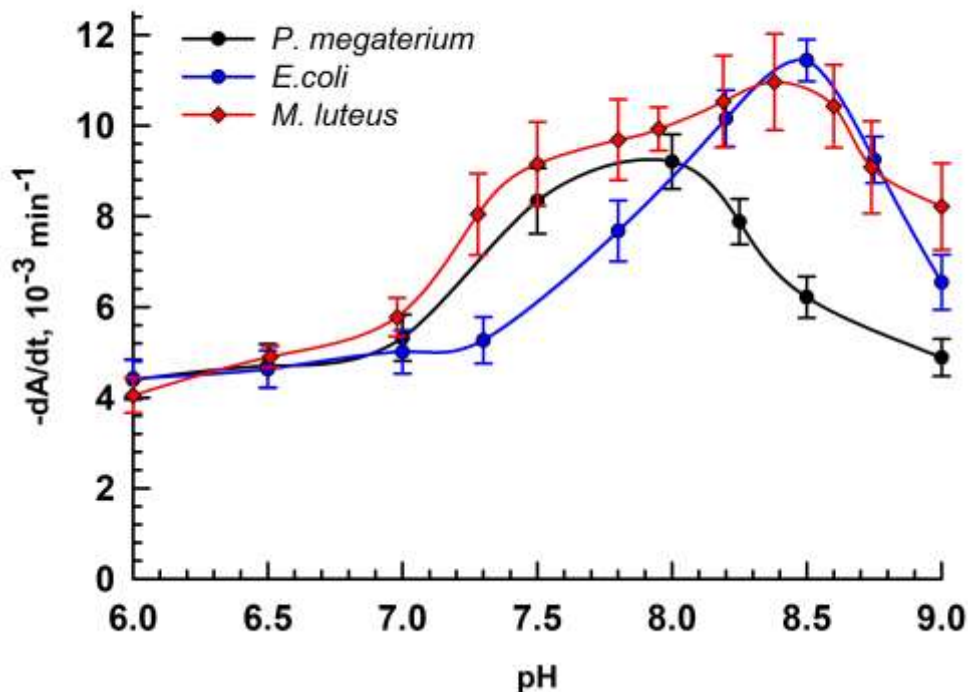
Measurement of lysozyme activity by means of the turbidimetric method [56, 57] was conducted in 0.01 M Tris-MES buffer solutions with addition of NaCl: 25 mM for *E. coli*, 55 mM for *P. megaterium*, 40 mM for *M. luteus*.

Registration of kinetic curves was carried out at a temperature of  $37^{\circ}\text{C}$ , at a wavelength of 650 nm. The initial absorbance of the cell suspension was 0.35—0.40. Before adding the enzyme to the cell suspension, a background decline of absorbance was registered for 90–120 s (and then subtracted when calculating the cell lysis rate), then after enzyme addition, the decline of absorbance was registered for 100—150 s. The initial lysis rate was obtained from the section of the kinetic curve from 15 to 120 s.

Every measurement was carried out for at least 7 times; the error value was calculated by means of the Student distribution with a confidence probability of 0.95.

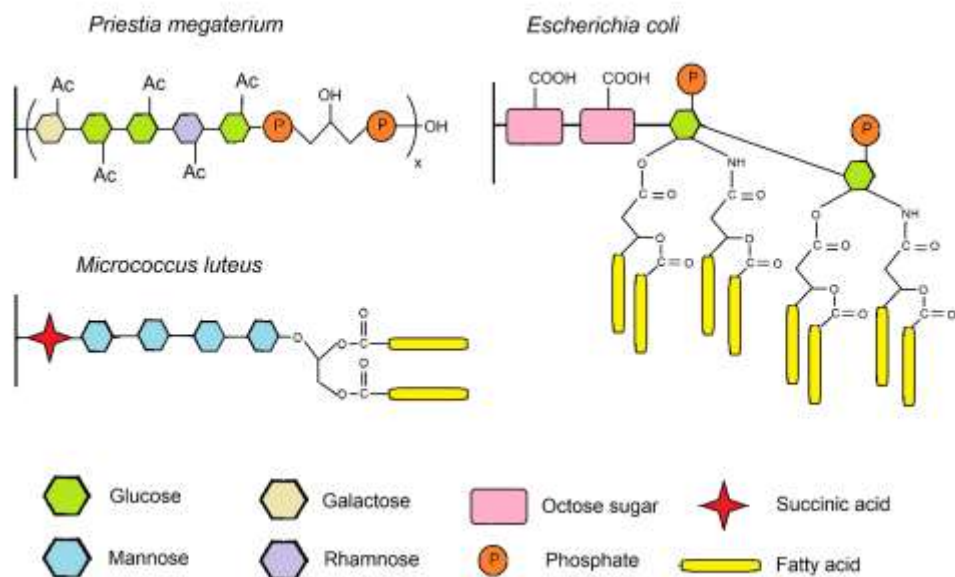
### 3 Results and discussion

Dependence of the rate of enzymatic lysis on pH in the range of pH 6.0–9.0 was obtained under conditions of linear dependence of the reaction rate on the concentration of the enzyme (lysozyme), and in the linear range of dependence of the analytical signal on the concentration of substrate (bacteria cells). As we can see, for all the species of bacteria considered, the pH dependence of the enzyme activity is bell-shaped, which is typical for most enzymatic reactions. But in this case, we observe a peculiarity - an insignificant dependence of the reaction rate on the pH value in the range outside the pH optimum, namely at pH from 6.0 to 7.0. The explanation of this fact may be the following: not only ionization of groups in the active site of the enzyme, but also presence of charged groups in the substrate (bacteria cells) can influence the effectivity of catalysis.



**Fig. 1.** The pH dependence of lysozyme activity towards *Priestia megaterium*, *Micrococcus luteus* and *Escherichia coli*. Concentrations of lysozyme are: 0.5 µg/mL for *P. megaterium*, 0.2 µg/mL for *M. luteus* and 0.1 µg/mL for *E. coli*. Initial absorbance of cell suspension is 0.42–0.45.

As we can see from the dependences obtained, the minimal resistance of bacteria, i. e. the maximal lysozyme activity, is observed at pH 8.0 for *P. megaterium*, 8.3 for *M. luteus*, and 8.5 for *E. coli*. Despite *E. coli* belongs to Gram-negative bacteria and *M. luteus* is Gram-positive, the optimal values of pH for lysozyme activity towards them are almost equal. This may be explained by the similarity in the structure of negatively charged compounds on the surface of *E. coli* and *M. luteus* cells. While *E. coli* tends typically to have a lipopolysaccharide (also known as endotoxin) at the outer membrane surface [58], on the outer surface of the peptidoglycan layer in *M. luteus*, instead of teichoic acids typical of gram-positive bacteria, a lipomannan is present, which is a lipopolysaccharide oligomer [59]. Unlike *M. luteus*, the negative charge of the cell wall of *P. megaterium* is provided by teichoic acids, similar to the teichoic acids of *Bacillus subtilis* [60, 61], but the closest analogue of them is a set of teichoic acids of some *Pneumococcus* strains [61, 62]. The influence of the structures described make pH dependences of cell lysis rate individual for each microorganism at a pH above 7.0, and in the range from 6.0 to 7.0 it affects the type of pH dependence equally.



**Fig. 2.** Structural schemes of the oligomeric components on the surface of the *Priestia megaterium*, *Escherichia coli* and *Micrococcus luteus* cell walls.

Our explanation on the influence of charges and hydrophobic groups of the cell wall of various bacteria on resistance to lysis matches and accomplishes investigations of cell wall surface properties by other methods. For instance, it was discovered by means of zeta-potential measurement that the surface of *E. coli* cell strains isolated from running water and sediment differs in charge [63]. The electrostatic behavior of the charge-regulated surfaces of Gram-negative *Escherichia coli* and Gram-positive *Bacillus brevis* was studied using numerical modeling in conjunction with potentiometric titration and electrophoretic mobility data as a function of solution pH and electrolyte composition [64].

As long as *Priestia megaterium* is widely applied as a probiotic culture, the information about its resistance of lysozyme can help us to choose the strain of *P. megaterium* itself and other components of commercial preparations designed for a definite pH value of the medium [65–67].

## 4 Conclusion

The research above demonstrates that the lowest resistance of bacteria to lysozyme is observed in the slightly alkaline pH region (8.0-8.6), but for different types of bacteria its range slightly differ, and in the pH range from 6.0 to 7.0, resistance to enzymatic lysis practically does not change for each of the microorganisms considered. The patterns observed are probably related to the structural features of negatively charged components of bacterial cell wall and outer membrane.

The results presented also should be considered when choosing methods of struggle against phytopathogenic and zoopathogenic bacteria strains by means of bacteriolytic enzymes.

The following research demonstrates the possibility of studying the resistance of many other bacterial species to enzymatic lysis using the turbidimetric method, which is many times superior to other existing methods of bacteria lysis in expressiveness and simplicity.



The research is performed in Lomonosov Moscow State University as a part of program: “Study of bacterial resistance to antibiotics based on the production of recombinant beta-lactamases, determination of their structure and interaction with substrates and inhibitors by mathematical modeling and enzymatic kinetics, development of antibacterial compounds and new drug forms, methods of molecular diagnostics of antibiotic resistance of bacteria and determination of antibiotics in environmental objects and food to create new approaches to effectively combat the resistance”.

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