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Screening of antagonistic bacteria against fungal phytopathogens

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Abstract

Water samples, bottom sediments and biofilms of lake were investigated to the microbial diversity. One hundred twenty-two isolated microorganisms were identified by cultural, morphological and biochemical characteristics. Bacterial strains were screened to evaluate their ability to produce amylase, catalase, lipase and cellulase enzymes. Aquatic bacterial strains are active producers of valuable exoenzymes with antimicrobial properties. Isolates demonstrated developed enzymatic complex and antifungal activity against phytopathogens - *Alternaria alternata, Botrytis cinerea, Fusarium solani, Fusarium oxysporum*. Most active and stable to enzymatic and antifungal activities strains were identified as: *Bacillus velezensis* (*Bacillus amyloliquefaciens*) and *Micrococcus yunnanensis* (*Micrococcus luteus*) by PCR-based molecular analysis. Due to results of antagonistic activity in this study aquatic bacteria are promising for application in agriculture sector.

Keywords: bacteria, activity, phytopathogen, enzyme.

1. Introduction

The water quality depends on pollutants (inorganic and organic) like chemical, biological, radiological, and physical. Organic water pollutants include organic pollutants from wastewater, fertilizers, pesticides, agricultural substances, forestry, food processing industry [15]. Water bacterial composition represents soil microorganisms formed from predominate strains also adapted to the localization conditions and water quality. Water pollution with organic substances are led by an increase in bacterial and fungal contamination. The same water microbial composition plays an important role in the process of self-purification from organic waste, which is used by the microorganism [14]. Lake ecosystems provide irreplaceable water resources for humans and microbiomes of the lake are suitable bioresources for the agriculture field. Microbial diversity of natural water ecosystems like lakes, and basins are a source of diverse microorganisms unique by their properties. Studies have shown that microbes identified in lakes are associated with water quality parameters and environmental conditions and their antimicrobial potential is promising for large application from the food industry to the pharmaceutical industry [3, 19, 23]. According to the literature, a lot of authors identified aquatic potential bacteria with high antagonistic activity due to developed enzymatic complex against pathogenic microorganisms which can be dangerous agents and cause many diseases.

Phytopathogenic fungi are mycotoxin producers caused to inflorescence disease, roots and seeds on agricultural crops and represent an important risk for humans. Fruit and vegetables production is greatly affected by major bacterial and fungal phytopathogens. Bacterial wilt can result in up to 100% yield losses. The most important fruit spoilage are caused by *Alternaria alternate, Alternaria solani, Botrytis cinerea, Fusarium sp.,* *Pseudomonas syringae*, *Xanthomonas vesicatoria* etc. Biologics based on antagonist bacteria are an alternative to chemical pesticides and an important element of a complete protection system in modern crop production, which allows for the reduction of the norms of the use of chemicals [21].

In this work, we isolated and identified bacteria from the lake with antagonist activity against fungal phytopathogens with the potential to be used in biological control of plant diseases.

2. Methodology

The strains of this study were isolated from the samples of water, bottom sediments and biofilms at the La Izvor Lake. Before each culture suspension was diluted until 10^{-8}, 10 g/ml of sample suspensions and 90 ml of sterile distilled water were prepared. For bacterial identification, dilutions were inoculated in plates with nutrient medium TSA, Kings B, SS agar, KIA, Endo and incubated at 28 °C for 72 hours.

To determine enzymatic activity, we used the method of inoculation on agarized selective media of different compositions. Bacterial isolates were screened using agar plates for testing amylase, catalase, celullase and lipase production, respectively. After incubation at 30°C for 24-48 hours, starch hydrolysis was determined by flooding the plates with iodine solution and hydrogen peroxide. Isolates showing zones of clearance were selected as enzyme-producing bacteria.

The fungal strains were grown in Petri plates on Saburo and malt agarized medium. The antifungal properties were determined using agar block method incubation at 30°C, 24-48 h. *Fusarium oxysporum CNMN-FF-06*, *Fusarium solani CNMN-FF-07, Alternaria alternata CNMN-FF-09, Botrytis cinerea CNMN-FF-10* have been used for antagonistic activity determination test, they are deposited in National Collection of Non-Pathogenic Microorganisms Institute of Microbiology and Biotechnology, Moldova.

Molecular techniques for identification were applied to isolated bacterial strains using PCR-amplification of the 16S rRNA gene and sequencing. The PCR conditions were: denaturalization for 3 min at 95 °C, 35 cycles of 30 min at 95 °C, 30 s at 53°C, 1 min at 72 °C and the final extension for 7 min at 72 °C, thereafter PCR products were cleaned following the manufacturer instructions. The PCR-products were sequenced in an Applied Biosystems 3500 Genetic Analyzer.

3. Results and discussion

One hundred twenty-two microorganisms were isolated from the water, biofilms and bottom sediments sampled of the lake. Isolates were identified based on the morphological, cultural and biochemical characteristics using different tests as: catalase, mannitol, citrate, urease, test CAMP, starch hydrolysis test, BEA test, nitrate reduction test, oxidase test, coagulase test, motar agar test, Methyl Red / Voges-Proskauer (MR/VP), test at MacConkey agar, sulphur reduction test (SIM). Preliminary analysis of phenotypic and morphological characteristics suggests the presence of the following microbial diversity: *Bacillus* sp., *Actinobacteria* sp, *Enterococcus* sp., *Micromyces* and *Pseudomonas* sp. The results of the microorganisms' identification from analyzed samples, based on the study of their cultural and morphological properties were supplemented after the study of biochemical characteristics as well as genetic analysis.

At result 65 strains were selected for further investigations to evaluate their ability to produce amylase, catalase, lipase, cellulase enzymes and antifungal activity. After adding iodine solution on plate, strains showed (or not) zones of clearance. Among the 65 bacterial strains 41,5% are possessed activities on three from four named enzymes, and 10,7% of the strains possessed activities to all four enzymes. The most of isolates have been shown amylase (48%) and catalase (61%) activity.

According to the literature, bacterial community is associated with water quality parameters and environmental conditions. Author Babich et al. studied the microbial diversity in water samples and bottom sediments of Lake Baikal and identified a widespread representative of the genera *Bacillus*, *Micrococcus*, *Acinetobacter* and phylogenetic groups of the genus *Pseudomonas*. Five strains were characterized as a source of metabolites with antimicrobial activity: *B. megaterium*, *P. fluorescens*, *P. aeruginosa*, *P. oleovorans P. putida*, later isolated metabolites were determined like bacteriocins. Researchers established that natural *Pseudomonas* isolates are active producers of exoenzymes, which are capable to the biodegradation process of simple and complex high-molecular compounds [1].

Extracellular hydrolytic enzymes participate in the mechanisms underlying fungal growth inhibition. Representatives of the *Bacillus, Pseudomonas, Actinobacteria* genus are characterized with antifungal activity against phytopathogens [2, 10, 17]. Author Majidzadeh et al. mentioned that *Actinobacteria* sp. strains isolated from soil are a source of antibacterial compounds and many other important secondary metabolites against test organisms [10]. Researchers Jiménez-Reyesa et al. also have carried out a study on phytopathogenic fungi - agents of plant diseases affected on plant during growth and storage period, moreover related to fruits and vegetables quality.

For antifungal properties determination were applied agar block method using phytopathogenic test-organisms *Fusarium oxysporum CNMN-FF-06, Fusarium solani CNMN-FF-07, Alternaria alternata CNMN-FF-09* , *Botrytis cinerea CNMN-FF-10* belong to the National Collection of Non-Pathogenic Microorganisms of the Institute of Microbiology and Biotechnology. Enzyme inhibition activities were measured by determining the diameter of the lysis zone. (Figure 1).

Figure 1. Antifungal activity of aquatic strains agains:
a) *Alternaria alternata* (strain 1 and 11); b) *Fusarium solani* (strains 42 and 57);
c) *Fusarium oxysporum* (strain 32 and 33); d) *Botrytis cinerea* (strain 61 and 62)

Diameter of the lysis zone ranged from 6,0±1,96 mm to 36,7±1,73 mm. Strain No. 1 showed the highest activity 30,0±2,26 mm against the *Alternaria alternata*, strain No. 57 have highest activity against the *Botrytis cinerea* 33,3±1,31 mm, strain No. 63 against the

Fusarium solani 23,0±1,13 mm, and strain No. 57 against the *Fusarium oxysporum* 33,0±1,31 mm.

Authors Maslienko et al. obtained the same results at the screening of antagonistic bacteria to the causative agent of soy fusarium and white rot of rapeseed [11, 12].

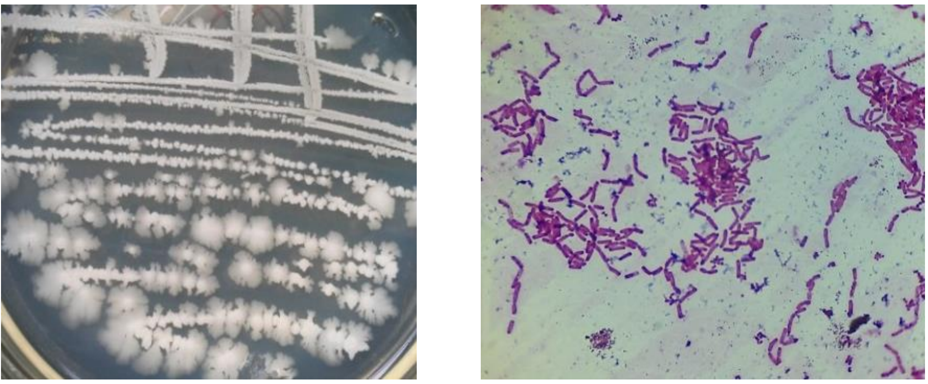
Fusarium solani and *Fusarium oxysporum* are agents caused vascular wilt diseases, rot damaging (after that colonize the xylem) in more than 100 plant species (Michielse and Rep 2009) [13].

Alternaria alternata is a latent fungus that develops during the cold storage of fruits and vegetables and caused black spots which become visible on the market period – losses for the economy and agriculture [18].

Botrytis cinerea or gray mold is one of the most widespread and destructive fungal diseases of horticultural crops therefore a lot studied plant pathogen which annual causes losses of $10 billion to $100 billion, resistance to a broad range of plant protection substances [22].

Different strains from water, bottom sediments and biofilms samples were selected for screening in the final molecular identification. The results obtained indicate that most of the Gram-positive bacteria are included in the *Bacillus* genus, specifically the 7 strain were identified *Bacillus velezensis* (*Bacillus amyloliquefaciens*). These strains shared about 99.5 % sequence similarity with regard to their 16S rRNA gene with *Bacillus velezensis* FZB42 and more than 99.4 % relatedness value with *Bacillus amyloliquefaciens* MPA 1034. Also identified *Micrococcus yunnanensis* (*Micrococcus luteus*) two strains shared on 99.7 % 16S rRNA gene sequence similarity with *Micrococcus luteus* DSM 200030 and on 99.9 % it DNA relatedness value with *Micrococcus yunnanensis* YIM 65004.

B. velezensis and *M. yunnanensis* isolated grow well in the nutrient agar medium. *M. yunnanensis* showed yellowish colonies after incubation at 37°C for 24 hr. By Gram-staining, both are Gram-positive, *B. velezensis* endo-spore forming, *M. yunnanensis* coccus non-spore formers arranged in tetrads and in irregular tetrads clumps (Figure 2).



a)

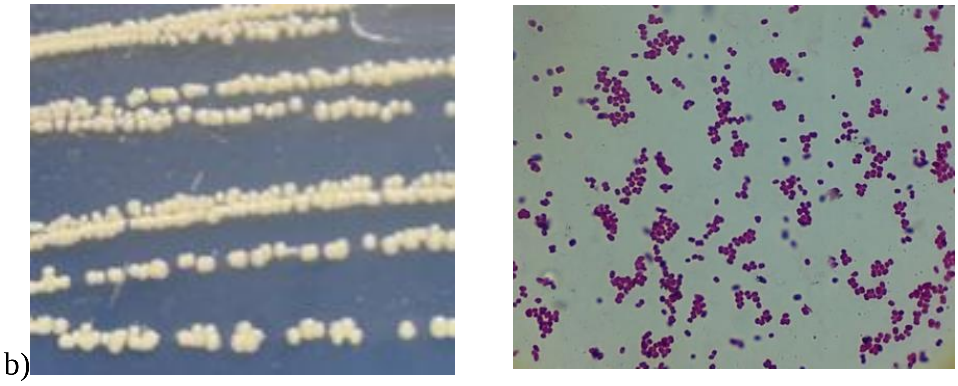


Figure 2. Morphological, cultural and tinctorial properties of *Bacillus velezensis* (a) and *Micrococcus yunnanensis* (b)

Based on these data and the lack of phenotypic distinctive characteristics, researchers Li-Ting Wang et al. and Fan et al. proposed *Bacillus velezensis* as a later heterotypic synonym of *Bacillus amyloliquefaciens* [5, 9]. Scientist Huda isolated and characterized the cartenoid pigment from *Micrococcus luteus* having antibacterial activity against five different bacterial isolates and antifungal activity against six different fungal isolates by well diffusion metho d, also having the ability to absorb UVA rays within the range of 300-500 nm [8].

Aerobic bacteria of the genus *Bacillus* have high antagonistic activity [20] and biological preparations based on it have significant advantages such as safety to humans and animals, resistance to adverse ecological factors, which makes it possible to use it effectively against plant diseases caused by phytopathogens of various nature [7]. Devi S. et al. mentioned *B. velezensis FZB42* is the most researched commercially used *Bacillus*-based biofertilizer and biocontrol agent in agriculture [4, 6].

Bacterial species *Bacillus amyloliquefaciens, Bacillus siamensis, Bacillus velezensis* and *Bacillus nakamurai* formed the *Bacillus amyloliquefaciens* operational group (OGBa). The representatives of OGBa are Gram-positive, endospore-forming, rod-shaped, widely distributed in soil, plants, food, water, also they are plant growth-promoting bacteria (PGPB) due to abilities to fix nitrogen, solubilize phosphate, produce siderophore and phytohormones as antimicrobial compounds for pathogens, produce amylase, protease, lipase, cellulase, xylanase, pectinase, aminotransferase, barnase, peroxidase enzymes and antimicrobial compounds [5, 16]. In this regard, genome analysis increased focus on the biotechnological applications of bacterial species belonging to the OGBa. For example, author Ngalimat et al. made a review on the biotechnological applications of the Operational Group *Bacillus amyloliquefaciens*, where highlighted *Bacillus* sp. for agriculture field [16].

4. Conclusions

A lot of studies showed the abilities of bacterial strains from natural source be important industrially and commercially. Our study indicated that La Izvor lake is of interest as a source of microorganisms like agents with promising biological control potential. The analysis of the obtained results confirmed that selected strains can be used for elaboration of new safety practices for the fungal phytopathogens control without using chemical compounds so prevent future soils and waters contamination. Screened strains will supplement the National Collection of Non-Pathogenic Microorganisms.

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References

1. Babich, O., Shevchenko, M., Ivanova, S., Pavsky, V., Zimina, M., Zimina, M., Noskova, S., Anohova, V., Chupakhin, E., Sukhikh, S. (2021) Antimicrobial potential of microorganisms isolated from the bottom sediments of Lake Baikal. *Antibiotics*, 10(8), 927. https://doi.org/10.3390/antibiotics10080927
2. Barreto, E. S. et al. (2008) Diversity in antifungal activity of strains of *Chromobacterium violaceum* from the Brazilian Amazon. *Journal of Industrial Microbiology and Biotechnology*. Volume 35, Issue 7, p. 783–790. https://doi.org/10.1007/s10295-008-0331-z
3. Byrsa, M., Cebotari, V., Burtseva, S. (2021) Presence of actinobacteria in the aquatic ecosystems of the “La izvor” lake system in the Chisinau city. Materials of the National Scientific Symposium with international participation “Modern Biotechnologies - solutions to the challenges of the contemporary world” Chisinau, Republic of Moldova, p. 41. https://doi.org/10.52757/imb21.007
4. Devi, S., Kiesewalter, H. T., Kovács, R., Frisvad, J. C., Weber, T., Larsen, T. O., Kovács, Á. T., Ding, L. (2019) Depiction of secondary metabolites and antifungal activity of *Bacillus velezensis* DTU001. *Journal Synthetic and Systems Biotechnology*, Volume 4, Issue 3, p. 142-149. ISSN 2405-805X, https://doi.org/10.1016/j.synbio.2019.08.002
5. Fan, B., Blom, J., Klenk, H-P., Borriss, R. (2017) *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* Form an “Operational Group B. *amyloliquefaciens*” within the *B. subtilis* Species Complex. Front. *Microbiol*. 8:22.
6. Fan, B., Wang, C., Ding, X., Zhu, B., Song, X., Borriss, R. (2019) AmyloWiki: an integrated database for *Bacillus velezensis* FZB42, the model strain for plant growth-promoting Bacilli. Database (Oxford). Jan 1; 2019: baz071. doi: 10.1093/database/baz071. PMID: 31219564; PMCID: PMC6585148.
7. Grabova, A. (2015) Skrining shtammov bakteriy roda *Bacillus* – aktivnykh antagonistov fitopatogenov bakterial'noy i gribnoy prirody / A. YU. Grabova, I. V. Dragovoz, L. A. Kryuchkova, L. A. Pasichnik, L. V. Avdeyeva // *Míkrobíologíchniy zhurnal* 77, № 6, 47-54
8. Huda, Z. Majeed Antimicrobial activity of *Micrococcus luteus* Cartenoid pigment. Al-Mustansiriyah. *Journal of Science*. Volume 28, Issue 1, 2017 . doi: http: //doi.org/10.23851/mjs.v28i1.31464
9. Li-Ting Wang, Fwu-Ling Lee, Chun-Ju Tai and Hsiao-Ping Kuo. (2008) Bacillus velezensis is a later heterotypic synonym of *Bacillus amyloliquefaciens*. *International Journal of Systematic and Evolutionary Microbiology*, 58, 671–675
10. Majidzadeh, M., Heidarieh, P., Fatahi-Bafghi, M., Vakili, M. (2021) Antimicrobial activity of Actinobacteria isolated from dry land soil in Yazd, Iran. *Mol Biol Rep*. 2021 Feb;48(2):1717-1723. doi: 10.1007/s11033-021-06218-y. Epub 2021 Feb 18. PMID: 33599952.

11. Masliyenko, L. V., Kurilova, D. A., Shipiyevskaya, Y., Asaturova, A. M. (2009) Pervichnyy skrining shtammov gribov i bakteriy antagonistov k vozbuditelyu fuzarioza soi *// Maslichnyye kul'tury*. №1 (140), 114–119
12. Masliyenko, L. V., Kurilova, D. A., Shipiyevskaya, Y. (2012) Skrining shtammov antagonistov vozbuditelya beloy gnili rapsa // *Nauch.-tekhnich. byull. VNIIMK*. Krasnodar, Vyp. 2 (151-152). 8 s.
13. Michielse, C., Rep, M. (2009) Pathogen profile update: *Fusarium oxysporum*. *Mol. Plant Pathol.* 10:311-324.
14. Mulamattathil, S.G., Bezuidenhout, C., Mbewe, M., Ateba, C.N. (2014) Isolation of environmental bacteria from surface and drinking water in mafikeng, South Africa, and characterization using their antibiotic resistance profiles. *J Pathog*. 2014:371208. doi: 10.1155/2014/371208 Epub 2014 Jul 6. PMID: 25105027; PMCID: PMC4106082.
15. Nasr, N., Arief, M., Hassan, A., Malhat, F. (2009) Persistent organic pollutants (POPs) in Egyptian aquatic environment. *Journal of Applied Sciences Research* 5:1929-1940.
16. Ngalimat, M.S., Yahaya, .S.R., Baharudin, M.M.A., Yaminudin, S.M., Karim, M., Ahmad, S.A., Sabri, S. A. (2021) Review on the Biotechnological Applications of the Operational Group *Bacillus amyloliquefaciens*. *Microorganisms* 9, 614.
17. Petatán-Sagahón, I., Anducho-Reyes, M. A., Silva-Rojas, H. V., Arana-Cuenca, A., Tellez-Jurado, A., Cárdenas-Álvarez, I.O,, Mercado-Flores, Y. (2011) Isolation of bacteria with antifungal activity against the phytopathogenic fungi *Stenocarpella maydis* and *Stenocarpella macrospora*. *Int J Mol Sci*. 12(9):5522-5537. doi:10.3390/ijms12095522
18. Rosalba, Troncoso-Rojas, Martín Ernesto, Tiznado-Hernández. In: *Postharvest Decay*, 2014
19. Slanina, V., Bogdan, N. (2021) Study of bacteria isolated from the bottom sediments of the lake La Izvor (in Romanian). Materials of the National scientific Conference with international participation "Integration through Research and Innovation" Moldova State University, p. 112-114.
20. Stadnichenko, M. A. (2011) Perspektivy biologicheskogo kontrolya vozbuditelya botritioza na paslenovykh kul'turakh // *Vestnik BGU* Ser. 2, 49-55.
21. Timofeyeva, M.G., Aktuganov, G.E., Krut'kov, V.M., Galimzyanov, N.F. (2011) Vliyaniye razlichnykh grupp sinteticheskikh pestitsidov na razvitiye, vyzhivayemost' i antagonisticheskuyu aktivnost' nekotorykh vidov aerobnykh sporoobrazuyushchikh bakteriy // *Izvestiya Samarskogo nauchnogo tsentra Rossiyskoy akademii nauk* 13, № 5, 239 -243.
22. Watkinson, S., Nicholas, M., Lynne, B. The Fungi (Third Edition). *Academic Press*, p. 245-292.
23. Yadav, N., Kour, D., Yadav, A.N. (2018) Microbiomes of freshwater lake ecosystems. *Journal of Microbiology & Experimentation*, Vol. 6, № 6, p. 245-248.