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Short Communication

Strain of *Penicillium* sp. Dag 2 isolated from the Caspian Sea, Russia

Eslanda Khalilova*[1], Nicolai Chernyh[2], Elvira Islammagomedova[1], Aida Abakarova[1], Dinara Aliverdieva[1]

[1]Precaspian Institute of Biological Resources of the Dagestan Federal Research Centre of the Russian Academy of Sciences, Makhachkala, Republic of Dagestan, Russian Federation.
[2]Vinogradsky Institute of Microbiology of the Russian Academy of Sciences, Moscow, Russian Federation.

Abstract: A strain of *Penicillium* sp. Dag 2 (PV250222) was isolated from seawater in the Caspian Sea (coastal area of the Samursky Nature Reserve, Republic of Dagestan, Russia). Morphologically, *Penicillium* sp. Dag 2 cells are spherical, 5.0×5.0 µm in size; they form glossy, convex, opaque, creamy-white colonies, 3–10 mm in diameter. Strain Dag 2 is a moderate halophile, tolerating up to 13.0% NaCl, and grows across a range of temperatures (0-10, 23-28, 30-37, and 35-40°C) and pH levels (5.6, 8.4-8.5). It utilizes monomeric sugars (L-glucose, D-mannose, D-sucrose, D-arabinose, D-xylose, D-lactose, D-maltose) as carbon and energy sources, as well as alcohols (D-sorbitol, D-mannitol, D-inositol, D-erythritol); does not produce indole or assimilate lysine or ornithine; capable of producing hydrolytic enzymes catalase, α-amylase, β-glucosidase, cellulase, lipase, pectinase, protease, xynalase; urease-, oxidase-, glucoamylase- and β-galactosidase-negative. The strain exhibited high sensitivity to the antibiotics nystatin, itraconazole, ketoconazole, clotrimazole, and fluconazole. *Penicillium* sp. Dag 2 demonstrated resistance to potassium tellurite and the pathogenic bacteria *E. coli*, *St. aureus*, and *Kl. oxytoca*. These studies enhanced our understanding of marine fungal ecology and distribution, and enabled the isolation of *Penicillium* sp. Dag 2, a strain with potential industrial applications.

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Introduction

In the International Code of Nomenclature for algae, fungi, and plants (ICN), fungi are recognized as a distinct branch of the tree of life (https://imafungus.biomedcentral.com). Marine fungi exhibit unique biochemical activity compared to terrestrial fungi, characterized by high osmotic tolerance and production of industrially valuable enzymes (Lyubakivskaya et al., 2024). As saprobes, symbionts, pathogens, or parasites, fungi contribute to the biogeochemical cycles of marine microbial ecosystems, inhabiting a wide range of substrates, including water, sponges, corals, algae, sand, and mangroves (Zhou et al., 2023). Fungal species of the genera *Aspergillus*, *Chaetomium*, *Cladosporium*, *Penicillium*, and *Trichoderma* occur in marine environments as facultative parasites, having developed morphological and physiological adaptations to extreme conditions. A *Penicillium* mold, identified as *Penicillium notatum*, was noted for inhibiting bacterial growth (Fleming, 1929). This discovery marked a public health milestone, and the identification of another key isolate, *P. chrysogenum* NRRL1951, later reclassified as *P. rubens* (Raper et al., 1944; Fierro et al., 2022), enabled the industrial production and widespread clinical use of penicillin. Since then, over 483 *Penicillium* species (hereafter referred to as *Penicillium* spp.) have been characterized (Houbraken et al., 2020). In recent decades, phenotype-based classification of *Penicillium* has been superseded by polygenic phylogeny, requiring DNA sequencing and marker analysis for accurate identification.

Penicillium fungi are recognized as valuable sources of novel natural products with diverse chemical structures and bioactive properties, offering potential for industrial and agricultural applications (Papikinou et al., 2024; Wadhwa et al., 2024).

*Correspondence: Eslanda Khalilova
E-mail: eslanda61@mail.ru
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Significant progress has been made in understanding the capacity of marine-derived *Penicillium* fungi to produce various enzymes and antimicrobial agents, which has contributed to the discovery of new drugs (Shaaban et al., 2023; Honghua et al., 2023; Agrawal et al., 2024; Du et al., 2024). Influenced by natural environmental conditions, marine *Penicillium* species are potential sources of over 452 unique bioactive compounds (He et al., 2023; Lv et al., 2024). Recent reviews have documented the characteristics of *Penicillium* species (Fierro et al., 2022; Zhou et al., 2023; Akaniro et al., 2023; Lv et al., 2024). This study reports the physiological, biochemical, and taxonomic properties of the *Penicillium* sp. Dag 2 strain, isolated from Caspian Sea water (Republic of Dagestan, Russia).

Materials and Methods

Objects of research and experimental area: The strain was isolated from a water sample collected in the Caspian Sea (coastal area of the Samursky Nature Reserve, Republic of Dagestan, Russia) in July 2022, 1km from the coast (41°48′57″N, 48°34′43″E). Water salinity ranged from 12.8 to 13.0‰, with temperatures between 21.9 and 25.5°C. Three samples were processed, yielding seven fungal cultures, with *Penicillium* species being minimally represented.

Physiological and biochemical analysis: Mold fungi were identified using standard guides (Visagie et al., 2014). Substrates for yeast cultivation media were weighed using a DV215CD analytical balance (Ohaus Discovery, Switzerland). Agar (Difco, Spain) was used for solid media preparation, with medium acidity adjusted to pH 4.5 using 1 N HCl or 4 M KOH (Russia) on a Hanna Instruments pH 211 pH meter (Germany).

The standard Czapek yeast autolysate agar was prepared as follows: Czapek concentrate 10 ml, sucrose 30 g, yeast extract (Difco) 5 g, K_2HPO_4 1 g, trace elements stock solution 1 ml, agar 20 g, dH_2O 1000 ml. Autoclaving at 121°C for 15 min, pH 6.2±0.2. Inoculations are made from spore suspensions in a semi-solid agar solution containing 0.2% agar and 0.05% Tween 80. All media are incubated at 25°C for 7 days, with additional CYA plates at 30 and 37°C. For microscopic observations, slides are prepared from 7-10 day-old colonies. The conidiophores have a simple form, characterized by solitary phialides. For medium with potassium tellurite (K_2TeO_3), 5 ml of a 2% salt solution was added per 1 L of medium. Primary isolation and culture incubation were performed in a Binder-115 microbiological incubator (USA) at 30 and 37°C for 1-7 days.

Morphological analysis and cell imaging were conducted using a CX21 light microscope (Olympus, Japan) and a Canon PowerShot A640 digital camera (Japan). Phenotypic properties, including cell and colony size, biochemical characteristics, oxygen and substrate utilization, temperature, and pH preferences, were assessed using standard methods (Pitt et al., 2000; Frisvad and Samson, 2004). Sensitivity to antifungal drugs and carbohydrate assimilation were evaluated using the disk diffusion method with standard disks ("Paper Indicator Systems for Identification of Microorganisms," Russia) containing 10-30 μg of antimicrobial agents, measuring growth inhibition zones per Methodological Guidelines MUK 4.2.1890-04.

Enzymatic activity was evaluated using express tests (MERCK, Germany) for the following enzymes: β-galactosidase (EC 3.2.1.23), using indicator disks impregnated with ortho-nitrophenyl-β-D-galactopyranoside (Conda, Spain); urease (EC 3.5.3.1), via CLO test; catalase (EC 1.11.1.6), with 3% H_2O_2 as substrate; oxidase (EC 1.4.3.3), using Kovacs reagent; amylase (EC 3.2.1.1) and pectinase (EC 3.4.11-15), detected with Lugol's reagent or starch-iodine; cellulase, using Congo red and Lugol's solution; phenylalanine deaminase (EC 4.3.1.24), with urea-PDA disks; and protease (EC 3.4.21), assessed by gelatin reactions. All enzymatic activity screening tests were conducted per the manufacturer's instructions. Yeast cultures were incubated at 30°C for 2 days in triplicate.

Phylogenetic analysis and statistics: Strain identification was conducted at the "Promyshlennyie Biotehnologii" Shared Use Research Center, Federal

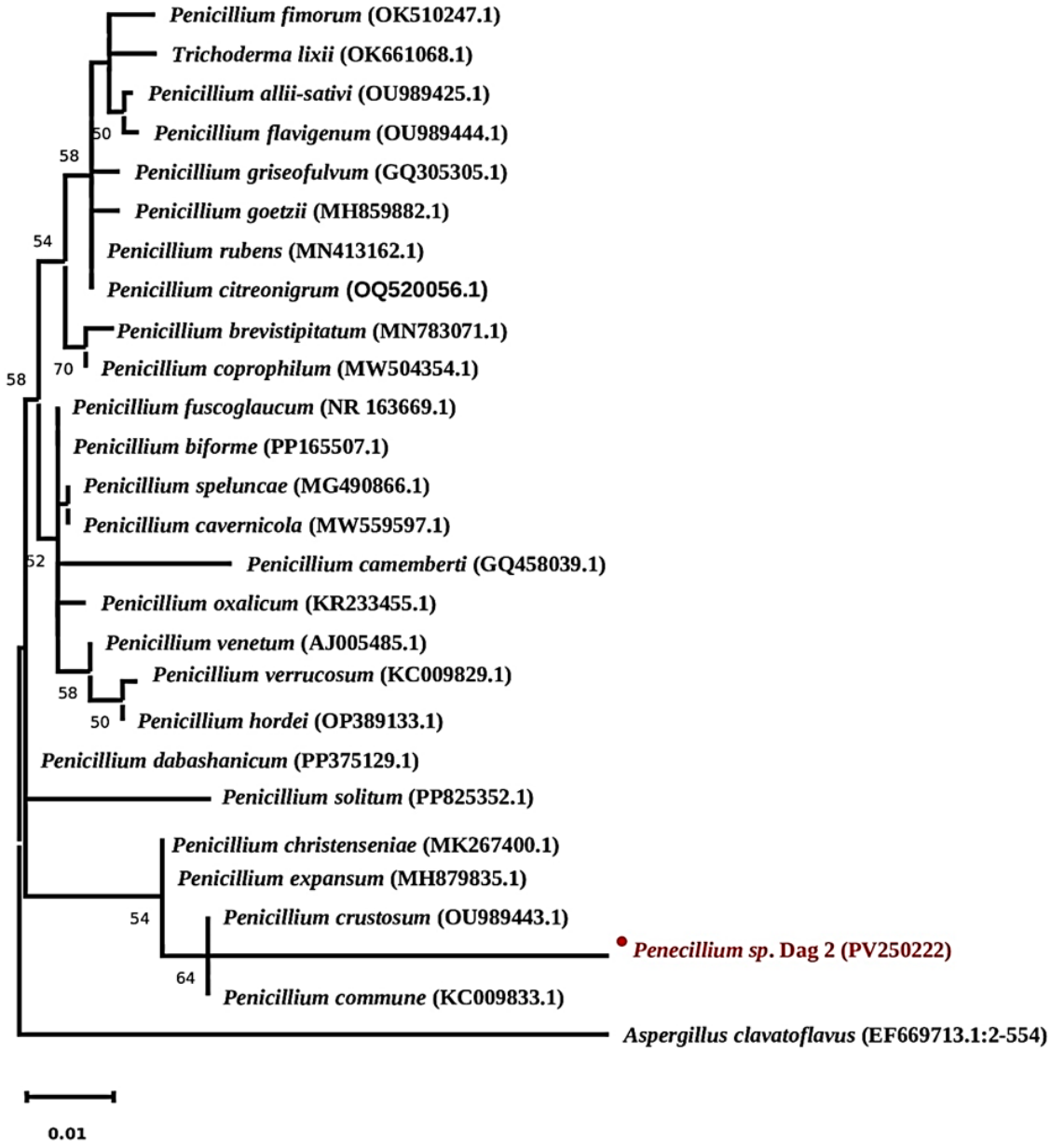

Figure 1. Position of *Penicillium* sp. Dag 2 (Gen Bank PV250222) on the phylogenetic tree constructed based on comparison of ITS DNA sequences (18S ribosomal RNA gene partial sequence internal transcribed spacer 1 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence and 28S ribosomal RNA gene partial sequence) and some representatives of the genus *Penicillium* using the maximum likelihood method. The sequence of *Aspergillus clavatoflavus* was chosen as an outgroup. The numbers indicate the number of bootstrap repetitions exceeding 50%.

Research Center for Biotechnology, Russian Academy of Sciences, Moscow. Polymerase chain reaction was performed using 18S rRNA primer systems (Kumar et al., 2018). PCR products were analyzed via electrophoresis on a 2% agarose gel at 6V/cm. PCR products were isolated and purified from low-melting agarose using the Wizard PCR Preps reagent kit (Promega, USA), following the manufacturer's instructions. Sequencing of ITS PCR fragments was carried out using the Sanger method (Sanger et al., 1977) with the Big Dye Terminator v.3.1 kit (Applied Biosystems, USA) on an ABI PRISM 3730 genetic analyzer (Applied Biosystems, USA). Amplifying and internal primers were used, with sequencing performed in both directions. Phylogenetic analysis of the 18S rRNA gene nucleotide sequences was conducted using the BLAST program in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). Sequence assembly and editing were performed using BioEdit

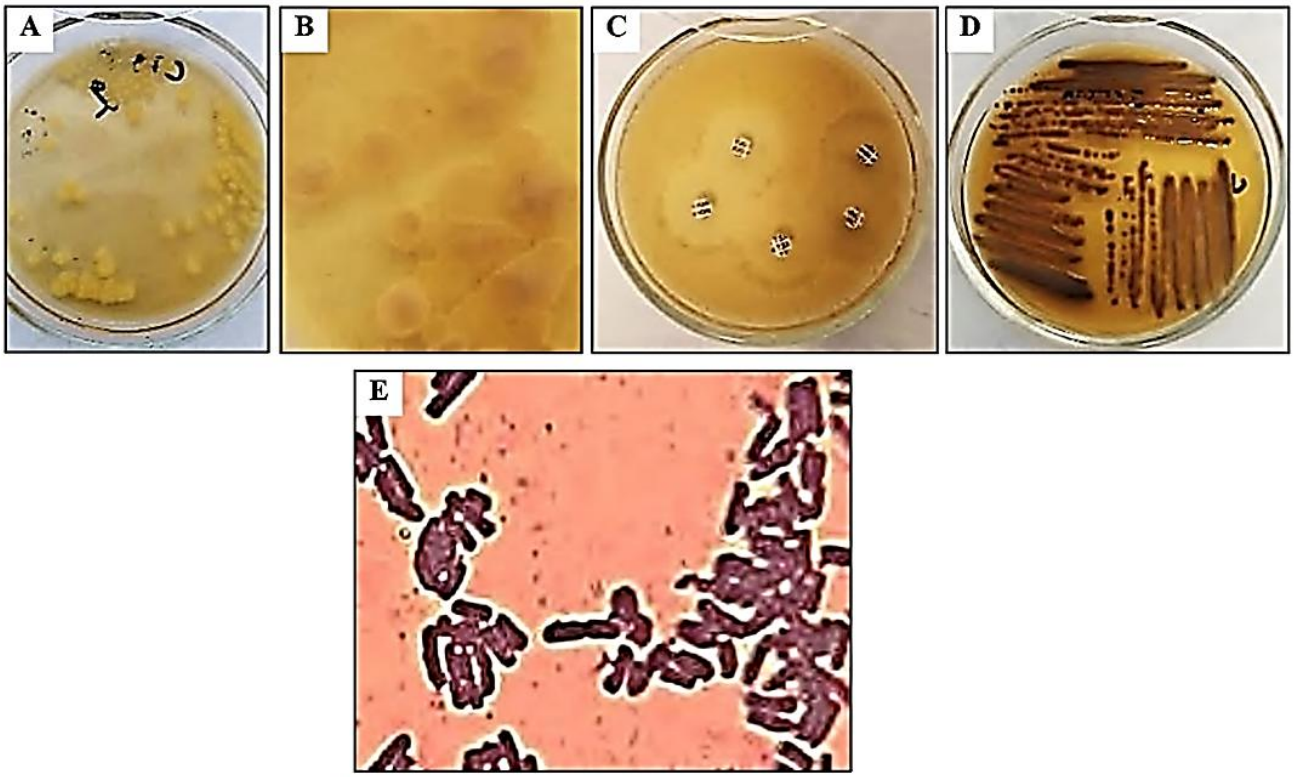

Figure 2. Morphological characteristics of *Penicillium* sp. Dag 2. Upper row (left to right): (A) On Czapek medium, 3 days, (B) on Czapek medium, 7 days, (C) on medium with potassium tellurite, (D) resistance to antibiotics nystatin, itraconazole, ketoconazole, clotrimazole, and fluconazole, and (E) Conidiospores.

(http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html).

The phylogenetic tree was constructed using the Maximum Likelihood method with the Tamura-Nei model (Tamura et al., 1993), employing Neighbor-Join and BioNJ algorithms to generate the pairwise distance matrix. Branch lengths were measured as substitutions per site. The analysis included 22 nucleotide sequences, encompassing 1st, 2nd, 3rd codon positions, and non-coding regions. Positions with less than 95% site coverage were excluded, and alignment gaps, missing data, and ambiguous bases were limited to less than 5%. Evolutionary analyses were conducted using MEGA X (Kumar et al., 2018).

To process the data we obtained, we used standard classical methods of analysis for testing statistical hypotheses. Calculations were performed using the «Function Wizard» in the «Statistical» category of Microsoft Excel. Differences with a p-value of ≤0.05 were considered statistically significant.

Results and Discussions

Genetic analysis of culture: Phylogenetic analysis of the ITS rDNA region positioned *Penicillium* sp. Dag 2 within the *Penicillium* cluster, showing a 64% confidence level with *P. commune* KC009833.1 (Spain), isolated from dry-cured ham, and 54% with *P. crustosum* OU989443.1 (Belgium) from air, *P. expansum* MH879835.1 (Pakistan) from fruits, and *P. christenseniae* MK267400.1 (USA) from yogurt as closest relatives (Fig. 1). Notably, *P. commune* is a key source of antipathogenic drugs for medical applications and biodegradation of oil waste, whereas *P. crustosum*, *P. expansum*, and *P. christenseniae* produce mycotoxins and indoles. The 18S rRNA gene fragment sequence of strain Dag 2 was deposited in the NCBI GenBank database under accession number PV250222.

Characteristics of the *Penicillium* sp. Dag 2 strain: Morphological analysis of *Penicillium* sp. Dag 2 revealed racemose clusters of branching conidiophores, intermediate branches, and spherical, smooth spores (5.0×5.0 µm in diameter), consistent with the morphological traits of the genus *Penicillium* (Frisvad and Samson, 2004). The Dag 2 strain is a

Table 1. Differentiating Characteristics of the Marine Strain *Penicillium* sp. Dag 2.

Phenotypic characteristics	*Penicillium* sp. Dag 2
Oxygen requirement	Facultative anaerobe
T, °C	0 – +10, 23-28, 35-40, 30-37
pH	4.5; 8.4-8.5
Pigmentation	Does not produce
NaCl Tolerance (%)	up to 13.0
Urease	–
Oxidase	–
Glucoamylase	–
β-galactosidase	–
β-glucosidase	+
Xinalase	+
α-amylase	+
Catalase	+
Lipase	+
Pectinase	+
Protease	+
Cellulase	+
Lysine	–
Ornithine	–
D-sorbitol	+
D-mannitol	++
D-inositol	+
D-erythritol	+
Indole	–
L-glucose	++
D-mannose	+
D-sucrose	++
D-maltose	++
D-arabinose	++
D-lactose	+
D-xylose	++
Antibiotic growth inhibition zone (mm)	
Ketoconazole	12
Clotrimazole	12
Fluconazole	11
Nystatin	14
Itraconazole	13
Resistance to pathogenic bacteria	
E. coli	+
St. aurens	+
Kl. oxitoka	+

moderate halophile, tolerating up to 13.0% NaCl. On Sabouraud agar, by day 7, it formed spherical, convex, opaque, yellow-pigmented colonies with a white border, 3-10 mm in diameter, and a dark reverse side (Fig. 2A, B).

The strain is psychrophilic (21.9-25.5°C), mesophilic (30-37°C), neutrophilic (pH 7.7-8.2), and acidophilic (pH 4.5). It utilized monomeric sugars (L-glucose, D-mannose, D-sucrose, D-arabinose, D-xylose, D-lactose, and D-maltose) as carbon and energy sources, as well as alcohols (D-sorbitol, D-mannitol, D-inositol, and D-erythritol). It did not produce indole or assimilate lysine or ornithine. The strain produced hydrolytic enzymes, including catalase (EC 1.11.1.6), α-amylase (EC 3.2.1.1), β-glucosidase (EC 3.2.1.21), cellulase (EC 3.2.1.4), lipase (EC 3.1.1.3), pectinase (EC 3.2.1.15), protease (EC 3.4.21–24), and xylanase (EC 3.2.1.8), but was negative for urease (EC 3.5.3.1), oxidase (EC 1.9.3.1), glucoamylase (EC 3.2.1.3), and β-galactosidase (EC 3.2.1.23).

The *Penicillium* sp. Dag 2 strain exhibited high resistance to the polyene antibiotic nystatin, triazole-group antibiotics itraconazole and fluconazole, second-generation synthetic antibiotic ketoconazole, and, to a lesser extent, imidazole-group antibiotic clotrimazole (Table 1, Fig. 2C). The strain's ability to detoxify absorbed tellurite was confirmed, with colony blackening indicating the reduction of tellurite to amorphous elemental tellurium (Fig. 2D), a key factor in bioremediation efficiency (Saghafi et al., 2020). *Penicillium* sp. Dag 2 showed resistance to pathogenic bacteria *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella oxytoca* (agar diffusion analysis) (Table 1).

Habitat: Seawater of the Caspian Sea (coastal area of the Samursky Nature Reserve, Republic of Dagestan, Russia).

Phylogenetic, morphological, and molecular analyses identified the isolated culture as *Penicillium* sp. Dag 2 strain. Genetic modifications in the strain, which alter its phenotype within the species, may be linked to environmental factors such as pollution by organic compounds and heavy metals, as well as the salinity of Caspian Sea water (Khalilova et al., 2023).

A notable feature of the isolate is its production of key hydrolytic enzymes—catalase, α-amylase, β-glucosidase, cellulase, lipase, pectinase, protease, and xylanase—which enable colonization of lignocellulosic substrates, algal biopolymers, marine snow, and plastics, facilitating microbial carbon sequestration and pollutant degradation in marine environments (Sen et al., 2022; Lyubakivskaya et al., 2024). The strain also exhibits resistance to pathogenic bacteria. The *Penicillium* sp. Dag 2 strain, isolated from the Caspian Sea coastal zone, exhibits significant potential for industrial applications, including the production of alcohol through grain

biopolymer conversion and the synthesis of industrially relevant enzymes.

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