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CONTRIBUTION TO THE STUDY OF CONSERVATION BY THE METHOD OF LYOPHILIZATION OF FUNGI AND BACTERIA

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Abstract

Microorganisms, especially filamentous fungi and bacteria, are a safe and inexhaustible source of potential producers of bioactive substances for agricultural use. The preservation and storage of these microorganisms plays an important role in biotechnology. There are different protective media for lyophilization of microorganisms, but this parameter is necessary to optimize in dependence of microorganism subjected to lyophilization. This study aims to elucidate the action of various protective media used in the process of lyophilization, strains of filamentous fungi and bacteria, isolated from aquatic basins, with high antimicrobial potential over a wide range of phytopathogens, on the viability and stability of antimicrobial properties after lyophilization. Four protective media were developed based on skimmed milk supplemented with glucose, sucrose and antioxidants in different concentrations. As a result of the research, the optimal protective medium for the lyophilization of bacteria and filamentous fungi was selected. After lyophilization in optimized media, the viability of bacteria varied within 88-92%, and of fungi 92-98%, compared to the initial titre, which ensures the preservation and long-term preservation of biotechnological interest strains.

Key words*: fungi, bacteria, protective medium, lyophilization.*

INTRODUCTION

Microorganisms are present in the endosphere, phyllosphere and rhizosphere of plants, in water and air, function effectively in a wide range of temperatures and represent a reliable and inexhaustible source of potential producers of biologically active substances for various areas (Brazhnikova et al., 2025; Harirchi et al., 2023; Kamal et al., 2018; Ratnam et al., 2014). Bacteria(Ayaz et al., 2023; Iqbal et al., 2024; Sahgal et al., 2024) and filamentous fungi (Armenova, 2025; Boughalleb-M'Hamdi et al., 2018; Al-Obaidy, 2019; Guzmán-Guzmán et al., 2025; Kumar et al., 2023) are widely used in the production of biopreparations for agricultural use. Plants are constantly exposed to various phytopathogens, which can significantly reduce crop yields. The use of chemical pesticides to control numerous crop diseases has negative impacts on the environment and human health. An alternative method of controlling phytopathogens, based on microorganisms, is now widely used. It is safer, less toxic, and reduces the severity of various crop diseases. These microorganisms, used as biocontrol agents for phytopathogens, also possess various capabilities, including the potential to stimulate plant development and growth, induce immune resistance in host plants, reduce pathogen infestation, degrade various types of hydrocarbons, and fertilize the soil (Ayaz et al., 2023; Mourou et al., 2022)**.**

The preservation and storage of microorganisms of industrial interest plays a vital role in biotechnology. Due to the widespread use of microorganisms in various industries, it is necessary to develop appropriate preservation and storage procedures to maintain their genotypic and phenotypic characteristics unchanged over time. Lyophilization is the most suitable method for preserving, storing, and transporting large collections of microbial cultures. During the lyophilization process, microorganisms are subject to various damages that lead to a loss of viability and cellular stability. This loss of viability is primarily caused by freezing, shock arising from dehydration, osmotic stress, low pressure leading to membrane rupture, fluctuations in cell membrane fluidity, loss of intracellular enzymatic activity, oxidation of fatty acids, and

loss of gene and protein function. In addition, the viability and stability of microorganisms during and after lyophilization and storage depend on factors such as cell density, freezing duration, residual moisture content after lyophilization, and the rehydration procedure (Li et al., 2011; Liu et al., 2019; Li et al., 2024; Morgan et al., 2006; Mputu Kanyinda, 2013).

To prevent harmful processes during lyophilization, a wide range of protective excipients has been developed. These can be classified as: colloids of animal, plant, or mineral origin; soluble substances such as sugars, peptones, and amino acids; and antioxidants. These components can be included in protective media individually or in a wide variety of combinations and serve as a medium for microbial cells, as well as ensuring osmotic balance and stress protection during freezing and sublimation (Okhapkina, 2009; Kaito et al., 2018; Rockingera et al., 2021).

Disaccharides are most often used as lyoprotectants in combination with protein products. Commonly used sugars include glucose (1% and 5%), and trehalose (4.74%, 5%, 7.5%, and 10%) (Li et al., 2024; Peiren et al., 2016; Wang et al., 2019), xylitol 1.45%, cellobiose 5%, D-galactose 5%, 7% and 10% sucrose (Bellali et al., 2020; Zhan et al., 2012), fructose 5% and lactose 7% (Wang et al., 2020).

The individual stages of the lyophilization process (freezing, primary drying, and secondary drying) expose proteins to various stresses. The use of appropriate excipients and control of the combined freezing and sublimation process can protect proteins during lyophilizetion (Izutsu, 2018). Trehalose is often used as an effective protectant due to its unique ability to displace water molecules tightly bound to carbonyl groups (Luzardo et al., 2000). Furthermore, the high viscosity and low mobility of trehalose may contribute to maintaining the integrity of microbial protein functions during lyophilization (Ohtake & Wang, 2011).

Dry skimmed milk is considered an ideal lyoprotectant, as it forms a viscous layer that increases the viscosity of the solution and prevents the growth of ice crystals during freezing (Castro et al., 1995; Suo et al., 2021). Skimmed milk powder is often used both alone and in combination with disaccharides, antioxidants, etc. (Valdez-Tenezaca et al., 2025). Thus, the combination of lactose 5.0% (w/v), mannitol 5.0% (w/v), trehalose 5.0% (w/v), skimmed milk powder 10.0% (w/v), ascorbic acid 0.5% (w/v) and gelatin 0.5% (w/v) was found to be the optimal choice for lyophilization of 18 marine strains belonging to the genera *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, *Planomicrobium*, *Edwardsiella*, *Enterococcus*, *Bacillus* and *Saccharomyces*, with the survival rate after lyophilization ranging from 2.3% to 95.1%. (Zhang et al., 2020).

Crowe & Crowe (2003) hypothesized that sugar combined with glass-forming excipients such as macromolecules would yield optimal results in the lyophilization of microorganisms. Polyols such as sorbitol and glycerol have also shown promise as cryopreservation excipients, but this success has not been translated to cell drying.

Lyophilization is considered a safe method for long-term preservation and storage of microorganisms, but to increase their survival and stability, it is necessary to use appropriate protective measures in this process.

The aim of this study is to determine the effect of various protective media used in the process of lyophilization of strains of filamentous fungi and bacteria isolated from water reservoirs and possessing high antimicrobial potential against a wide range of phytopathogens on the viability and stability of antimicrobial properties after lyophilization.

MATERIALS AND METHODS

The objects of the study were 10 strains of filamentous fungi (5 strains of *Trichoderma* spp. and 5 strains of *Penicillium* spp.) and 6 strains of bacteria represented by the genera: *Bacillus* sp. (4 strains), *Pseudomonas* sp. (1 strain) and *Micrococcus* sp. (1 strain), isolated from water ponds in the Chisinau Municipality, Republic of Moldova.

Lyophilization of microorganisms

For lyophilization of fungi and bacteria, four protective media were developed: 1 - skimmed milk + 5% sucrose + 5% glucose + 0.01% ascorbic acid; 2 - skimmed milk + 7% glucose + 0.1% KI; 3 - skimmed milk + 10% glucose +

0.1% ammonium molybdate; 4 - skimmed milk + 5% glucose + 5% glycerol + 0.01% ascorbic acid. Skimmed milk + 7% glucose was used as a control protective medium for fungi, and skimmed milk + 7% sucrose was used for bacteria.

The lyophilization process consists of freezing and vacuum sublimation. Freezing was performed in the Ult Freezer 80 refrigerator at a temperature of -50ºC. Sublimation of the samples was performed in the sublimation system "LABCONCO 6 plus" (temperature - 92ºC, pressure 6 Pa, duration 8 hours).

The titer of the spore suspensions of the studied strains was determined before lyophilization. The following phytopathogens were used as test strains: *Alternaria alternata*; *Botrytis cinerea*; *Fusarium solani*; *Fusarium oxysporum*; *Agrobacterium tumefaciens*; *Corynebacterium michiganensis*; *Erwinia carotovora* and *Xanthomonas campestris*.

After lyophilization in various protective media, the strains were activated with sterile distilled water and subcultured in Petri dishes on agar media, after which their viability was assessed. The antimicrobial activity of *Penicillium* sp. 1, *Penicillium* sp. 6, *Trichoderma* sp. 3, *Trichoderma* sp. 14, and *Bacillus* sp. 13, which demonstrated high antagonism to the reference strains before lyophilization, was assessed after lyophilization.

The viability of the strains before and after lyophilization (expressed in colony-forming units (CFU) per mL^{-1}) was determined by counting colonies on agar medium after serial dilutions. The number of viable cells was expressed as the *log*10 of colony-forming units (CFU) in 1.0 mL of suspension. Viability was calculated using the formula BSR = (*log*AL/*logB*L) × 100, where BSR is the strain survive rate in %, *log*BL is the logarithm of the CFU count before lyophilization, and *log*AL is the logarithm of the CFU count after lyophilization or storage (Muñoz-Rojas et al., 2006).

The antimicrobial activity of microorganisms was determined using the disc-diffusion method (Egorov, 2004; Ratnam et al., 2014).

The tested cultures were subcultured in Petri dishes. The 8 mm agar blocks were cut with a sterile cork borer from the nutrient substrate where the strains of tested microorganisms grew abundantly. The agar blocks were then transferred to prepared cavities in agar nutrient medium with instantly subcultured tests. Petri dishes were kept in a cool place for 1 hour before incubation to allow the diffusion of biocidal substances. The diameter of the growth inhibition zones was measured after incubation at 37°C for 24 h for bacteria, and at 28°C for 72 h for fungi, respectively.

There were three replications for each test (was applied to the significance threshold p=0.05) and the biocidal assessment was performed twice. Statistical calculations were performed using MS Excel 2010 software.

RESULTS AND DISCUSSIONS

Viability of microorganisms before and after lyophilization

Protective media used in the lyophilization process have different effects on microorganisms, and a universal protective medium has not yet been developed.

In the process of lyophilization, dehydration of microorganisms occurs, and as a result, the oxidative state of the cells increases and the production of reactive oxygen species (ROS), free radicals. An imbalance between ROS and antioxidants or enzymes, which capture peroxide, can lead to peroxidation of lipids, proteins and nucleic acids (França et al., 2007; Len et al., 2019; Van Engeland et al., 2025). Furthermore, the osmotic imbalance resulting from lyophilization (freezing and sublimation) leads to water loss, which seriously impacts cell membranes by altering the physico-chemical properties of the lipid bilayer. Although organisms employ various adaptive strategies under dehydration conditions, immediate adaptation to osmolytes (OMA), such as sugars and amino acids, is critical for maintaining membrane stability (Van Engeland et al., 2025).

The following substances are often used as lyoprotectants for lyophilization of microorganisms: skimmed milk, trehalose, and monosodium glutamate, while water and skimmed milk are used as rehydration media. Carbohydrates, glycerol, and antioxidants (ascorbic acid, thiourea, potassium iodide, and ammonium molybdate) are used to enhance the growth effect and stabilize the protective

medium (Okhapkina, 2009; Pajuelo et al., 2023; Rockingera et al., 2021; Van Engeland et al., 2025).

When assessing the viability of strains of *Penicillium* fungi (Figure 1) after lyophilization in the developed protective media, it was found that the viability of 5 strains varies significantly depending on the composition of the protective media used.

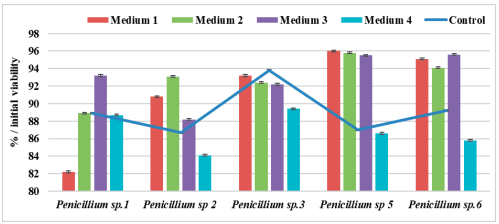

Figure 1. Viability of *Penicillium* strains after lyophilization in different protective media compared to the initial titre

The viability of the lyophilized strains in the control medium varied within the limits of 86-92%, compared to the initial viability, of the strains that were lyophilized in Medium 1 was 90.8-96%, of the strains in Medium 2 - 88.9-95.5%, of those that were lyophilized in Medium 3 - 88.2-95.6%, and of those lyophilized in Medium 4 - 84.1-89.4%, compared to the initial titre before lyophilization. Thus, we can see that the strains lyophilized in 3 elaborated protection media (Medium 1; Medium 2 and Medium 3) demonstrated a higher viability than the initial one, compared to the control variant, with the exception of the strains lyophilized in protection Medium 4, in which the viability was lower than the control. In most strains, the highest viability was obtained in the variant in which protection Medium 1 was used, which contains: SM + 5% S + 5% G + 0.01% Aa.

The evaluation of the viability of *Trichoderma* strains after lyophilization in the elaborated protection media demonstrated a higher viability, compared to *Penicillium* strains (Figure 2).

The viability of 5 *Trichoderma* strains after lyophilization in the control protective medium was 91.6-95.6% compared to the initial titer before lyophilization. After lyophilization in protective Medium 1 (SM + 5% S + 5% G + 0.01% Aa), the viability of *Trichoderma* strains ranged from 93.8% to 97.3%, and for lyophilized strains in protective Medium 2, the viability was 92.1-96.6% compared to the initial viability.

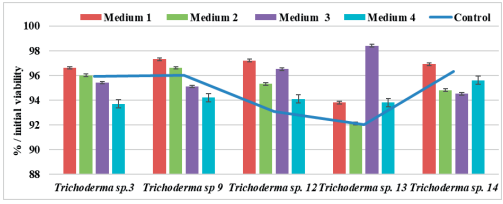

Figure 2. Viability of *Trichoderma*strains after lyophilization in different protective media compared to the initial titre

High viability was recorded for *Trichoderma* strains lyophilized in Medium 3. Thus, for 4 strains, viability was in the range of 94.5-96.5%, and for the strain *Trichoderma* sp. 13, viability was 98.0% compared to the initial viability before lyophilization. In the variant in which the strains were lyophilized in protective Medium 4, viability was 93.7-95.6% compared to the original. The highest viability after lyophilization of *Trichoderma* strains was obtained in the variant in which medium 1 containing (SM + 5% S + 5% G + 0.01% Aa) was used as a protective medium.

According to the obtained results, the viability of fungal strains after lyophilization in the protective medium M1 is higher compared to the viability of lyophilized strains in the control protective medium (SM +7% G).

After lyophilization in various protective media, bacterial strains demonstrated high viability in all tested variants (Figure 3).

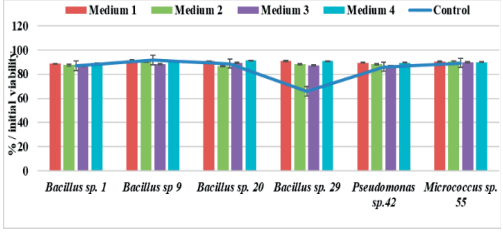

Figure 3. Viability of bacteria strains after lyophilization in different protective media compared to the initial titre

The viability of 6 bacterial strains after lyophilization in the control medium (SM + 7% S) varied within 65.5-89% compared to the

viability before lyophilization. When lyophilized in the developed protective media, their viability increased compared to the control variant. Thus, in the variant with protective Medium 1, the viability was 88.5-91.2% compared to the initial. In the variant that used protective Medium 2, the recorded viability varied within 86.5-90.1%. After lyophilization in protective Medium 3, the recorded viability values were within 86.6-89.6%, and in the variant with protective medium 4, the bacterial strains demonstrated the highest level of viability - 89.1-91.5% compared to the initial viability before lyophilization.

The results demonstrated higher viability of fungal and bacterial strains after lyophilization in variants M1 and M4, in which the protective medium was supplemented with ascorbic acid, a powerful antioxidant essential for reducing oxidative stress. These results are consistent with literature data showing that certain compounds, such as glucose, trehalose, and ascorbic acid, can reduce lipid peroxidation and improve cell survival (Pajuelo et al., 2023; Rodklongtan et al., 2022; Van Engeland et al., 2025).

Rodklongtan et al. (2022) demonstrated that the addition of a small amount of ascorbic acid to lactose-based medium could promote cell viability during lyophilization, but the effect on viability became negative with increasing concentration.

Antimicrobial activity of some strains before and after lyophilization

As a result of studying the antimicrobial properties of the strains before lyophilization, 4 fungal strains (*Penicillium* sp. 1, *Penicillium* sp. 6; *Trichoderma* sp. 3 and *Trichoderma* sp. 14) and 1 bacterial strain (*Bacillus* sp. 13) with high antimicrobial potential againsttest strains were identified.

Filamentous fungi of the genera *Tichoderma* and *Penicillium* are successfully used in agriculture as biological control agents, phytostimulants for agricultural plants, in soil bioremediation, etc. (Mirsam et al., 2025; Sun et al., 2025; Woo et al., 2014).

To assess the stability of the antimicrobial properties of the specified strains after lyophilization, the antimicrobial activity of the lyophilized strains was determined in 4 developed protective media, as well as in a control protective medium.

The antimicrobial activity of *Penicillium* strains before lyophilization revealed zones of inhibition of test fungi ranging from 18 mm to 31 mm, while the zones of inhibition of phytopathogenic bacteria ranged from 16.0 to 18.7 mm. Evaluation of antimicrobial activity after lyophilization revealed a decrease in the zones of inhibition of phytopathogens by 1-3 mm compared to the initial zone before lyophilization. Thus, under the influence of *Penicillium* spp. 1 (Figure 4) and *Penicillium* spp. 6 (Figure 5), lyophilized in a control medium, the inhibition zone of test fungi varied from 16 to 29 mm. In the variant where *Penicillium* strains were used after lyophilization in protective Medium 1, the inhibition zones of phytopathogenic fungi ranged from 17 mm (*F. solani*, *F. oxysporum*) to 30 mm (*A. alternata*), and for strains lyophilized in protective Medium 2, protective Medium 3 and protective Medium 4, the inhibition zones of control fungi ranged from 15 mm to 27 mm.

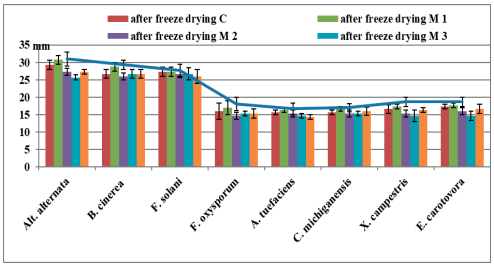

Figure 4. Antimicrobial activity of *Penicillium* sp. 1 strain before and after lyophilization in different protection media

Testing of *Penicillium* strains after lyophilization in the developed protective medium on test bacteria showed inhibition zones from 15 to 17 mm, regardless of the variant tested.

Compared with the variant in which the control protective medium was used, the highest antimicrobial activity against the test strains was recorded for the strains *Penicillium* sp. 1 and *Penicillium* sp. 6 in the variant in which the strains were lyophilized in protective medium 1 (SM + 5% S + 5% G + 0.01% Aa), and the lowest in the variant with protective medium M 3.

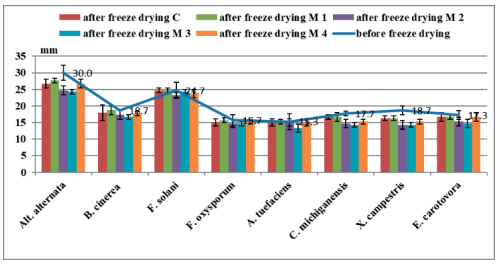

Figure 5. Antimicrobial activity of *Penicillium* sp. 6 strain before and after lyophilization in different protection media

Testing of *Trichoderma* sp. 3 (Figure 6) and *Trichoderma* sp. 14 (Figure 7) strains on reference fungal strains before lyophilization showed high activity: inhibition zones from 20.7 mm (*F. oxysporum*) to 39 mm (*A. alternata*), and against phytopathogenic bacteria, the inhibition zones ranged from 12 to 17 mm.

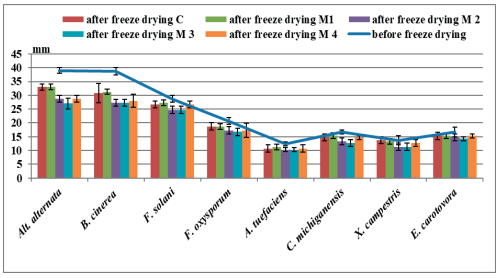

Figure 6. Antimicrobial activity of *Trichoderma* sp. 3 strain before and after lyophilization in different protection media

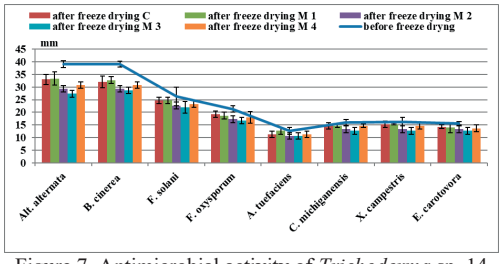

Figure 7. Antimicrobial activity of *Trichoderma* sp. 14 strain before and after lyophilization in different protection media

After lyophilization in various protective media, the inhibition zones of phytopathogens decreased. Thus, under the influence of strains in the control variant, the zones ranged from 15 mm (*F. oxysporum*) to 31 mm (*A. alternata*), under the influence of strains lyophilized in protective medium 1, the zones were 18–32 mm, for strains lyophilized in protective medium 2, the zones ranged from 17 to 29 mm. Lyophilized strains on medium 3 demonstrated zones of inhibition of phytopathogenic fungi ranging from 17 to 27 mm, while lyophilized strains on medium 4 demonstrated zones of inhibition of phytopathogenic fungi ranging from 17 to 30 mm. Compared to the reference bacteria, the zones of inhibition were small and did not exceed 15 mm, regardless of the *Trichoderma* strain variant tested.

The highest antimicrobial activity compared to the control variant was recorded under the action of exometabolites of the mentioned strains, lyophilized in protection Medium 1.

According to the obtained results, it was established that the inhibition zones of reference strains (phytopathogenic fungi and bacteria) are larger under the influence of exometabolites of *Trichoderma* strains lyophilized in protective medium 1, but still 2-3 mm smaller compared to the data obtained before lyophilization of the indicated strains.

Bacteria of the genus *Bacillus* play a crucial role in the prevention and control of plant diseases, producing a wide range of compounds with antifungal properties, such as isocoumarin and amino acids, bacillomycin, bacteriocins, iturin, surfactin, cyclic lipopeptides, polyketides, lantibiotics, phospholipids, polyketide microlectins (Iqbal et al., 2024; Zhang et al., 2008).

The *Bacillus* sp. 13 strain before lyophilization, demonstrated antagonism against 3 phytopathogenic fungi with inhibition zones of 20-22 mm and against 2 phytopathogenic bacteria with inhibition zones of 17.7-21.7 mm (Figure 8).

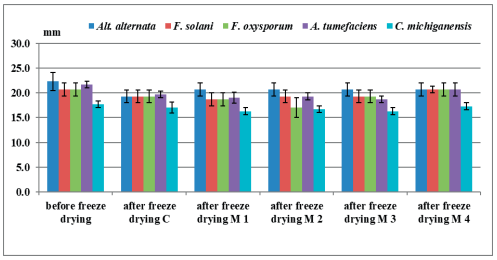

Figure 8. Antimicrobial activity of *Bacillus* sp. 13 strain before and after lyophilization in different protection media

Under the influence of exometabolites of the *Bacillus* sp. 13 strain (Figure 8), lyophilized in

the control medium (SM + 7% S), the inhibition zones of phytopathogens: *A. alternata*, *F. solani* and *F. oxysporum* were 19 mm, and the inhibition zones under the influence of the lyophilized strain in protective Medium 1, Medium 2 and Medium 3 varied from 17 mm to 20 mm. The larger inhibition zones were obtained under the influence of the lyophilized strain in protective medium 4, which were 20-22 mm.

Compared with the test bacteria *A. tumefaciens* and C. *michiganensis*, regardless of the medium in which lyophilization was carried out, the *Bacillus* sp. 13 strain demonstrated inhibition zones of 17-20 mm.

CONCLUSIONS

Lyophilization is a widely used method of long-term preservation, in which the survival of microorganisms depends significantly on the protective medium used.

Testing of five protective media on fungal and bacterial strains during lyophilization revealed minor but significant differences depending on the type of protective medium used for lyophilized cultures ($p < 0.001$).

Among the tested media, the highest viability and antimicrobial activity after lyophilization of filamentous fungal strains was obtained in a protective medium containing skimmed milk + 5% sucrose + 5% glucose + 0.01% ascorbic acid, while for bacterial strains, the highest viability was achieved in a medium containing skimmed milk + 5% glucose + 5% glycerol + 0.01% ascorbic acid.

Cell viability of mycelial fungi after lyophilization ranged within 92-98%, and of bacteria within 88-91.5%

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