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Metschnikowia pulcherrima as a Biocontrol Agent against Potato (*Solanum tuberosum*) Pathogens

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Abstract: An increasing trend in protecting plants against phytopathogens is the replacement of chemical pesticides with environmentally acceptable biopreparations. This article focuses on the possible use of yeast *Metschnikowia pulcherrima* as a biocontrol agent against potato pathogens. The scope included an assessment of the antimicrobial activity of 10 *M. pulcherrima* isolates against 10 phytopathogens: *Fusarium oxysporum*, *Fusarium sambucinum*, *Rhizoctonia solani*, *Alternaria solani*, *Alternaria*, *tenuissima*, *Alternaria alternata*, *Colletotrichum coccodes*, *Phoma exigua*, *Pectobacterium carotovorum*, and *Streptomyces scabiei*, by the agar-well diffusion method. Pulcherrimin formation, enzymatic profiles detected by the API ZYM system, and metabolite formation evaluated by HPLC analysis were conducted for the most active *M. pulcherrima* isolates. Leucine arylamidase, valine arylamidase, α- and β-glucosidase, and esterases were the most noteworthy in the pattern of activity. In turn, ethanol, glycerol, and organic acids (acetic, succinic, lactic acids) were determined in the largest quantities. The isolate *M. pulcherrima* TK1 was selected and cultured on supplemented acid whey. An in situ experiment was carried out on the seed potatoes, which showed a 30%–100% reduction in nine phytopathogens; only *P. carotovorum* was insensitive to yeast treatment. Therefore, *M. pulcherrima* TK1 was proposed as the potential biological solution for seed potato protection against phytopathogens.

Keywords: biopesticide; antifungal activity; acid whey; pulcherrimin; enzymatic activity

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1. Introduction

The potato (*Solanum tuberosum* L.), which is grown on about 18.6 million hectares in 150 countries and yields 322 Mt annually, is the world's fourth-most-important crop, after maize (637 Mt), rice (585 Mt), and wheat (549 Mt), and is consumed by billions of people [1]. Apart from its efficient yield per unit area, potato tubers are a valuable source of fibre, carbohydrates, and minerals such as calcium, potassium, magnesium, copper, and iron [2]. Leading countries in world potato production are China and India [3], whereas, in the European Union, about three quarters (76.8%) of the cultivated area of potatoes is concentrated in just six countries: Poland (21.6%), Germany (16.5%), France (12.9%), Romania (10.0%), the Netherlands (9.9%), and Belgium (5.9%).

Potato tubers' post-harvest management is a large problem due to the invasion of pathogens. The sources of infections are frequently contaminated crop residues in the field after previous cultivation. Such residues can lead to diseases during storage caused by fungal agents, such as *Fusarium*, *Alternaria*, and *Rhizoctonia* genera. Dry rot of seed

tubers, which results in crop losses up to 25%, is caused by *Fusarium* strains [4], whereas the presence of *Alternaria* spp. can lead to early blight, marked by dark spots with concentric rings on the leaves [5]. The latter, a soilborne fungus *Rhizoctonia*-related disease, is widely spread and destructive for the crops, lowering the yield by 30% or even 70% in the northern continental climate [6].

Farmers tend to apply several pesticides, including metalaxyl, mancozeb, and fluopicolide, to combat phytopathogens. These chemically synthesised chemicals have a powerful antimicrobial efficacy and represent numerous adverse effects on the natural environment. They have the potential to penetrate deep into the soil, disrupt microorganism populations, and pollute groundwater when in use [7–9]. These compounds irritate the skin and respiratory mucous membranes in humans, and their cytotoxic and genotoxic effects are also known [10]. Therefore, the European Union is moving away from chemical plant-protection products, especially in organic farming, which accounts for 13% of all potato holdings [11]. This approach to agricultural production prioritises environmental and wildlife conservation, avoiding synthetic chemicals such as fertilisers and pesticides in favour of biological and mechanical means, as well as cultural approaches such as hand weeding, pruning, and mulching [12].

Given the expected rise in pest outbreaks and the possible breakdown of present biological control agents as a result of global climate change, alternative techniques to biological control are required. Using augmentative releases of natural enemies to maintain high concentrations of biological control agents is one method for mitigating future pest damage [13]. Using biological control agents specific to an invading pest is an alternative for invasive pest control [14].

Metschnikowia yeasts can be considered as a potential agent for seed potato protection against phytopathogens. *Metschnikowia* yeasts are part of the natural microbiota of fruits, juices, and flower insects [15]. Their antagonistic effect is not connected with the production of toxins. *Metschnikowia* yeasts, apart from a typical mode of action, such as competition for nutrients and space, can secrete pulcherriminic acid, which forms a complex with Fe^{3+} ions, called pulcherrimin [16]. That is why the aggressive action of the yeast is based on the sequestration of iron—a crucial element for the growth of pathogens. In addition, *Metschnikowia* yeasts can produce extracellular lytic enzymes (chitinase and glucosidases), release microbial volatile compounds, and form a biofilm that supports the antifungal effect [16–19]. Recently, Sipiczki [20] proposed merging these species able to produce red pigment—pulcherrimin—into one unit, the clade of *M. pulcherrima*. Antimicrobial properties of *M. pulcherrima* yeasts against phytopathogens have been described in several works [18,21–23]; however, they mainly concern fruits.

Therefore, this work aimed to assess the potential application of yeasts *Metschnikowia* as a biocontrol agent against various potato pathogens. The most active yeast strain was selected, and then the growth medium was optimised based on the acid whey. The biocontrol activity of the selected strain was also assessed in situ on potatoes. This is the first report on the biocontrol potential of *M. pulcherrima* yeasts against potato phytopathogens.

2. Materials and Methods

2.1. Microorganisms Used in the Research

2.1.1. Yeasts from the *Metschnikowia* Clade

Eight isolates of *M. pulcherrima* clade from Polish fruits and flowers and two collection strains of *M. pulcherrima* from the National Collection of Yeast Cultures (Norwich, UK) were used in the research, in order to select the most effective antimicrobial agent against potato phytopathogens. Yeasts were cultivated on YPD agar (BTL, Łódź, Poland) for 72 h at 25 °C and then stored at 4 °C. All of the tested strains are presented in Table 1.

Table 1. *Metschnikowia* yeasts used in the research.

Strain	Origin
Metschnikowia pulcherrima NCYC 747 *Metschnikowia pulcherrima* NCYC 2321	National Collection of Yeast Cultures (Norwich, UK)
Metschnikowia pulcherrima J2*	Isolate from apple fruit
Metschnikowia pulcherrima J3*	Isolate from apple fruit
Metschnikowia sp. J4	Isolate from apple fruit
Metschnikowia pulcherrima J6*	Isolate from apple fruit
Metschnikowia pulcherrima TK1*	Isolate from strawberry flower
Metschnikowia sp. M3	Isolate from raspberry fruit
Metschnikowia sp. TO1	Isolate from strawberry fruit
Metschnikowia pulcherrima M4*	Isolate from raspberry fruit

* species identification by MALDI-TOF mass spectrometry; confidence score value range 96.2%–99.9%.

Identification of *Metschnikowia* sp. isolates was conducted using the MALDI-TOF MS method. Five most active strains of *Metschnikowia* sp. were grown at 25 °C for 24 h on Malt Extract Agar (MEA) (Merck, Darmstadt, Germany) plates and analysed with the AXIMA-iD Plus Confidence MALDI-TOF MS system (Kratos Analytical Ltd. and Shimadzu Corporation, Kyoto, Japan) and SARAMIS PREMIUM software (Spectral ARchive And Microbial Identification System, bioMérieux, Marcy l'Étoile, France). The manufacturer's direct smear plus formic acid method was employed. Briefly, microbial colonies were spread on dedicated, analytical metal plates with a sterile 1 µL inoculation loop. Then, 0.5 µL of 25% formic acid (FA) was spotted onto cells, mixed well, and left until almost dry. Finally, 1 µL of saturated α-cyano-4-hydroxycinnamic acid (α-CHCA) solution in acetonitrile:ethanol: water (1:1:1 *v*/*v*) mixture containing 3% trifluoroacetic acid (TFA) was added onto the disrupted microbial cells, mixed well again, and air-dried at room temperature. The mass spectra were acquired and processed using Launchpad 2.9 software (Kratos Analytical Ltd. and Shimadzu Corporation, Kyoto, Japan) in the SARAMIS linear positive mode, with a laser frequency of 50 Hz in the mass-to-charge ratio (*m*/*z*) ranging from 2000 to 20,000 Da (laser power 90, 200 per sample; 5 shots accumulated per profile) for each mass spectrum. *E. coli* DH5α (TAKARA BIO INC.) cells were used as a calibrator of the AXIMA-iD Plus Confidence MALDI-TOF MS system and internal control of the identification process, in accordance with the recommendations of the manufacturer.

The results of the comparative analysis of obtained spectra with the reference spectra in the SARAMIS database were expressed as confidence score values (%) with associated colour codes. The score values highlighted in dark green were considered as excellent ID (>99.9% ID confidence), in light green and in yellow as good ID (from 90% to 99.8% and from 85.0% to 89.9% ID confidence, respectively), and in white as sufficient ID (with 70.0% to 84.9% ID confidence), as the analysed spectrum was sufficiently close to that of the reference spectrum. Results lower than 70% were automatically set to fail and were also shown in white, with 0% of the ID confidence.

2.1.2. Potato Phytopathogens

Pectobacterium carotovorum PCM 2056 was acquired from the Polish Collection of Microorganisms of the Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences (Wrocław, Poland). *Alternaria alternata* ŁOCK 408 was obtained from the Collection of Pure Cultures of Industrial Microorganisms ŁOCK at the Lodz University of Technology (Łódź, Poland). *Phoma exigua* DSM 62040, *Fusarium sambucinum* DSM 62397, *Colletotrichum coccodes* DSM 62126, *Rhizoctonia solani* DSM 22843, *Alternaria tenuissima* DSM 63360, and the bacterial species *Streptomyces scabiei* DSM 40778

were purchased from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). *Fusarium oxysporum* Z154 and *Alternaria solani* Z184 isolates were kindly provided by Jadwiga Śliwka from the Plant Breeding and Acclimatization Institute (IHAR)—National Research Institute (Radzików, Poland).

Strains were cultivated on tryptic soy agar (TSA) (Merck, Darmstadt, Germany) for bacterial strains and on potato dextrose agar (PDA) (Merck, Darmstadt, Germany) for moulds and stored at a temperature 4 °C. The suspensions of fungal strains were prepared by collecting spores from the surface of the pure culture on the PDA medium and resuspending in 0.85% NaCl with Tween (0.02%), for a final concentration of 10^6 CFU/mL. Bacterial suspensions were prepared from the pure culture on the TSA medium and adjusted to a final concentration of 10^6 CFU/mL.

2.2. Agar-Well Diffusion Method for In Vitro Antimicrobial Activity Determination

The agar-well diffusion method was used to perform the in vitro assessment of yeasts' antimicrobial activity against potato phytopathogens [24]. *Metschnikowia* strains were cultured on YPD medium (for preliminary screening) or different variants of supplemented acid-whey-based media (for final selection of cultivation medium) for 72 h at a temperature of 25 °C on a shaker (Unimax 1010, Heidolph, Germany) at 160 rpm. Then, 100 μL of freshly prepared phytopathogens suspension were inoculated onto the PDA plates for mould strains and the TSA plates for bacterial strains. Next, 10 mm diameter wells were cut by a sterile cork bore, and 250 μL of yeast cultures were transferred into the wells. The plates were incubated at 25 °C for 2–5 days, depending on the phytopathogen. YPD and supplemented acid-whey-based media not inoculated with phytopathogens were used as control samples. The diameters of pathogen growth-inhibition zones were measured manually, excluding the well width. The results were expressed as the average of three independent repetitions for each *Metschnikowia* strain—phytopathogen combination with a standard deviation value.

Simultaneously, yeast cultures on YPD were filtrated through syringe filters (pore diameter 0.22 μm) and assessed in terms of antimicrobial activity, as described above.

2.3. Assessment of Pulcherrimin Production Profile by Selected Strains of Yeast Metschnikowia

Selected strains of *Metschnikowia* strains were cultured on YPD agar plates, supplemented with three different concentrations of $FeCl_3$ (Chempur, Piekary Śląskie, Poland), 0.005, 0.01, and 0.2 mg/mL, for 96 h at temperature 25 °C. Then, the diameter of the coloured halo around the colonies was manually measured and expressed as an average of three independent repetitions with a standard deviation value. Additionally, the colour of colonies from the top and the bottom of the plate was assessed on a scale: white/creamy (no pigmentation), pink, red, and dark red.

2.4. Determination of the Enzymatic Activity of Selected Strains of Yeast Metschnikowia by API ZYM Test.

Enzymatic profiles of selected strains of *Metschnikowia* yeast for 19 enzymes was assessed using API ZYM system (bioMérieux, Marcy l'Étoile, France), in accordance with the instructions of the manufacturer.

2.5. Fermentation Byproducts of Selected Metschnikowia Yeast Strains by High-Performance Liquid Chromatography (HPLC)

Organic acids and alcohols used as standards were purchased from Supelco (Bellefonte, PA, USA). Sulphuric acid (95%–98%) obtained from J.T. Baker B.V. (Deventer, Holland) was a "Baker instra-analyzed" reagent. HPLC-grade water was obtained using an Aquinity E60 Lifescience TI system (membraPure GmbH, Bodenheim, Germany). A Finnigan Surveyor liquid chromatograph with PDA and RI detectors (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for the research. The compounds were separated

using an Aminex HPX 87 H+ column (300 × 7.8 mm) from Bio-Rad (Hercules, CA, USA). The eluent was 0.005M H_2SO_4. The elution rate was 0.6 mL/min; the detection was carried out in the range of 200–350 nm (for the PDA detector) for 40 min [25]. The acids were determined by comparing the retention times and UV spectra with a standard substance, alcohols (glycerol and ethanol), by comparing the retention times [25].

2.6. Optimisation of the Acid-Whey-Based Culture Media for Cultivation of Metschnikowia Pulcherrima TK1

Optimisation of the supplemented acid-whey-based culture media for cultivating *Metschnikowia pulcherrima* TK1 was conducted on a microscale using Multiskan GO Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA) equipped with SkanIt RE for Multiskan GO 3.2 software (Thermo Scientific, USA). Then, 200 μL of 43 different variants of supplemented acid-whey-based media listed in 2.7. were transferred into 96-well plates and inoculated with 50 μL of *M. pulcherrima* culture. The control samples were YPD and Yeast Extract Peptone—YP (1% of yeast extract, 2% of peptone; BTL, Łódź, Poland). The plates were incubated for 72 h at a temperature of 25 °C, while the turbidity was measured every hour at a wavelength of 600 nm. The experiment was performed in triplicate. Biomass yield (Yx) was calculated according to Equation 1:

$$Yx = A_{max} - A_{min} \quad (1)$$

where A_{max} is the maximum absorbance value of inoculated culture medium, A_{min} is the minimum absorbance value of inoculated culture medium. The maximum specific growth rate (μ_{max}) was calculated according to Equation 2:

$$\mu_{max} = (\ln A_{max} - \ln A_{min})/\Delta t \quad (2)$$

where A_{max} is the maximum absorbance value of inoculated culture medium, A_{min} is the minimum absorbance value of inoculated culture medium, and Δt is the time difference.

2.7. Acid-Whey-Based Media for Yeast Cultivation

The acid whey was acquired from JOGO—Łódź Dairy Cooperative (Kraszewo, Poland). The supplements were yeast extract (BTL, Łódź, Poland) and peptone (BTL, Łódź, Poland) for 16 different variants of growth medium and, additionally, glucose (BTL, Łódź, Poland) for the following 27 variants of the acid-whey-based medium. Acid whey supplementation is presented in Table 2.

Table 2. Variants of acid whey supplementation.

Medium Variants	Yeast Extract (%)	Peptone (%)	Glucose (%)	Medium Variants	Yeast Extract (%)	Peptone (%)
Y50 P50 G50	0.5	1.0	1.0	Y100 P100	1.0	2.0
Y50 P50 G25	0.5	1.0	0.5	Y100 P50	1.0	1.0
Y50 P50 G12.5	0.5	1.0	0.25	Y100 P25	1.0	0.5
Y50 P25 G50	0.5	0.5	1.0	Y100 P12.5	1.0	0.25
Y50 P25 G25	0.5	0.5	0.5	Y50 P100	0.5	2.0
Y50 P25 G12.5	0.5	0.5	0.25	Y50 P50	0.5	1.0
Y50 P12.5 G50	0.5	0.25	1.0	Y50 P25	0.5	0.5
Y50 P12.5 G25	0.5	0.25	0.5	Y50 P12.5	0.5	0.25
Y50 P12.5 G12.5	0.5	0.25	0.25	Y25 P100	0.25	2.0
Y25 P50 G50	0.25	1.0	1.0	Y25 P50	0.25	1.0
Y25 P50 G25	0.25	1.0	0.5	Y25 P25	0.25	0.5
Y25 P50 G12.5	0.25	1.0	0.25	Y25 P12.5	0.25	0.25
Y25 P25 G50	0.25	0.5	1.0	Y12.5 P100	0.13	2.0
Y25 P25 G25	0.25	0.5	0.5	Y12.5 P50	0.13	1.0

Y25 P25 G12.5	0.25	0.5	0.25	Y12.5 P25	0.13	0.5
Y25 P12.5 G50	0.25	0.25	1.0	Y12.5 P12.5	0.13	0.25
Y25 P12.5 G25	0.25	0.25	0.5			
Y25 P12.5 G12.5	0.25	0.25	0.25			
Y12.5 P50 G50	0.13	1.0	1.0			
Y12.5 P50 G25	0.13	1.0	0.5			
Y12.5 P50 G12.5	0.13	1.0	0.25			
Y12.5 P25 G50	0.13	0.5	1.0			
Y12.5 P25 G25	0.13	0.5	0.5			
Y12.5 P25 G12.5	0.13	0.5	0.25			
Y12.5 P12.5 G50	0.13	0.25	1.0			
Y12.5 P12.5 G25	0.13	0.25	0.5			
Y12.5 P12.5 G12.5	0.13	0.25	0.25			
YPG	1	2	2			

2.8. Characteristics of the Chemical Composition of Selected Acid-Whey-Based Media

YPD, pure acid whey, and selected Y12.5 P12.5 G50 acid-whey-based medium were analysed. Iron (test no. 8008, FerroVer), potassium (test no. LCK228), ortophosphates (test no. 8048, PhosVer), chloride (method no. 8113) and ammonium nitrogen (test no. 8038, modified Nessler method) concentrations were determined using DR6000 spectrophotometer (HACH-LANGE, Loveland, CO, USA), in accordance with the instructions of the manufacturer. Lactose and lactic acid analysis was performed using enzymatic kits (Megazyme Ltd., Bray, Ireland), in accordance with the procedure of the manufacturer, and a UV-spectrophotometer (Thermo Scientific Multiskan Go, Thermo Fisher Scientific, Munich, Germany). The Kjeldahl method was used to assess the protein concentration [26]. Calcium and sodium were determined with flame photometers BWB-XP (BWB Technologies, Newbury, UK). Samples for phosphorus and nitrogen were mineralised using an LT200 thermoreactor (HACH-LANGE, Loveland, CO, USA). Total solids and volatile solids were assessed using standard methods [27].

2.9. In Situ Assessment of Antimicrobial Activity on Seed Potatoes

'Impresja' seed potatoes were acquired from Zamarte Potato Breeding (Zamarte, Poland). In situ antimicrobial assay on potatoes was conducted according to the method previously described in Steglińska et al. [28,29] research. Briefly, seed potatoes were cut 5 mm deep and 5 mm wide with a cork bore. Then, potatoes were dipped (about 500–2500 mL/kg potatoes, depending on the dipping container) in 72 h cultures of *M. pulcherrima* TK1 (about 10^7 CFU/mL) for 5 min and inoculated with 20 μL of each bacterial or fungal phytopathogen's suspension (about 10^6 CFU/mL in 0.85% NaCl) in the cuts. Control samples were only inoculated with the phytopathogens' suspension, and potato samples were incubated for 14 days at a temperature of 25 °C. The percentage of phytopathogen infestation was measured in comparison to the controls, according to Equation 3:

$$\text{Inhibition (\%)} = (C - T)/C \times 100 \quad (3)$$

where C is the percentage of phytopathogen infestation on control potatoes, T is the percentage of phytopathogen infestation on potatoes treated with yeast cultures. The percentage of the infested area was measured after cutting the seed potatoes in half. The test was conducted in triplicate.

2.10. Statistical Analysis

Statistical analysis was performed with Statistica 13.1 (Statsoft, Tulsa, OK, USA). The antimicrobial activity (phytopathogen growth-inhibition zones) of *Metschnikowia* yeasts and the production of fermentation byproducts was compared within the sample using a one-way analysis of variance (ANOVA) at a significance level of 0.05. When a statistical

difference was detected ($p < 0.05$), the means were compared using Tukey's post hoc procedure at a significance level of 0.05.

3. Results and Discussion

3.1. Antimicrobial Activity of Metschnikowia Yeasts In Vitro

The potential use of *Metschnikowia* yeasts as an antimicrobial agent for plant protection against phytopathogens has already been widely described for *Penicillium expansum* [30–33] and *Botrytis cinerea* [18,34,35] biocontrol for fruits. These pathogens are mainly responsible for the spoilage of apples, grapes, pears, and strawberries. The potato biocontrol by *Metschnikowia* yeasts has not been of interest to researchers so far. This study is the first comprehensive report on the antimicrobial activity of *Metschnikowia* yeasts on potato pathogens in vitro.

The results are presented in Figure 1. The majority of the tested yeast cultures demonstrated a broad spectrum of antifungal activity. No antibacterial activity of *Metschnikowia* strains was, however, observed. The ability of *Pectobacterium carotovorum* to cause potato soft rot is regulated by the phenomenon of quorum sensing [36,37]. This could be the reason why this pathogen was insensitive to the yeast treatment. The solution would be to look for quorum sensing inhibitors, in order to inhibit *P. carotovorum* development. Environmental isolates turned out to be more active than collection strains, in terms of both spectrum of activity and diameter of inhibition zones. *Rhizoctonia solani* and *Fusarium sambucinum* were the most susceptible pathogens; the growth-inhibition zones were equal to at least 7.0 ± 0.0 mm and 7.0 ± 1.0 mm, respectively. *Phoma exigua* and *Colletotrichum coccodes* presented the highest resistance to collection strains of *Metschnikowia* yeast and isolates marked with J2, J4, TK1, TO1, and M3 symbols (Figure 1). Several works reported the antifungal activity of *Metschnikowia* yeast against notorious plant pathogen *A. alternata* [18,22,38,39] in in vitro experiments, and our work confirmed this observation. The growth-inhibition zones of this fungi ranged from 5.0 ± 0.0 to 8.0 ± 0.0 for the *Metschnikowia* isolates in the present study. Gore-Lloyd et al. [40] noted antifungal activity against *F. oxysporum*, which was also inhibited in our work by all tested *Metschnikowia* strains. No antimicrobial activity of the culture filtrates without yeast cells was observed, which confirms the Spadaro et al. [41] observation that living cells of *M. pulcherrima* yeasts are necessary for their biocontrol abilities.

Overall, *Metschnikowia* isolates marked with J2, J3, J6, TK1, and M4 symbols were identified as the most effective in potato biocontrol in vitro, based on their activity spectrum as well as the diameter of their inhibition zones, and selected for further experiments (Figure 1).

3.2. Determination of Pulcherrimin Formation, Enzymatic Activity, and Fermentation Byproducts of Metschnikowia Pulcherrima

The antimicrobial action of *Metschnikowia* yeasts is often described as a result of iron depletion by pulcherrimin formation. This observation is compatible with Gore-Lloyd et al.'s [40] work, where pulcherrimin-producing *Metschnikowia* strains were found to be more active against moulds than pigmentless mutants. Pulcherriminic acid, the secondary metabolite of *M. pulcherrima* clade, forms a chelate complex with iron ions present in the environment, which causes iron to be unavailable for other microorganisms. This chelate complex, called pulcherrimin, turns yeast colonies light pink to dark red, depending on the Fe^{3+} concentration in the medium. When secreted extracellularly, pulcherrimin forms coloured halos around the colonies [20]. Pulcherrimin itself does not exhibit antimicrobial activity [39].

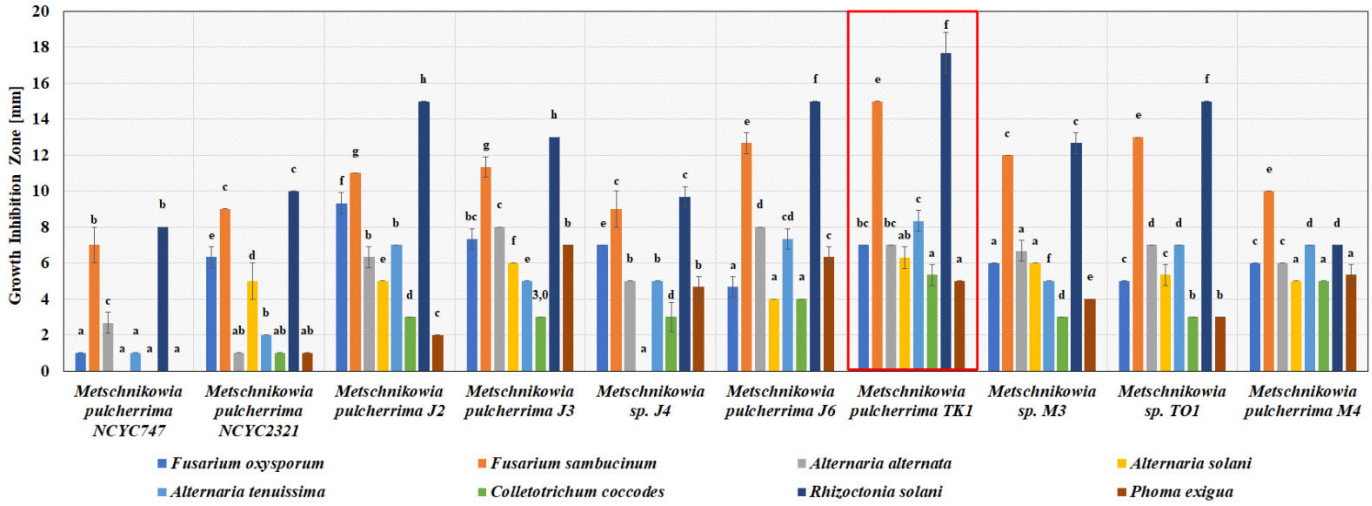

Figure 1. The antimicrobial activity of *Metschnikowia* sp. strains cultivated on YPD medium against potato pathogens, measured as growth-inhibition zones in the agar-well diffusion method. *Pectobacterium carotovorum* and *Streptomyces scabiei* are not presented in the figure due to the lack of *Metschnikowia* strains antimicrobial activity against these phytopathogens. Culture filtrates (without cells) are not presented in the figure due to the lack of antimicrobial activities. a–h results, with different letters within the sample, are significantly different (Tukey's test, $\alpha = 0.05$).

In our work, pulcherrimin formation for the selected five *M. pulcherrima* strains was assessed on YPD plates supplemented with three different concentrations of $FeCl_3$ (0.005, 0.01 and 0.2 mg/mL). The colony colour and diameter of halos for each yeast strain are presented in Table 3 and Figure 2. The halos diameter reached the widest level (1–6 mm) in plates with 0.005 mg/mL $FeCl_3$ supplementation and decreased further as the $FeCl_3$ concentration increased to 0.01–0.2 mg/mL. As the halos became smaller after exceeding 0.01 mg/mL $FeCl_3$ supplementation, the colony colour turned red due to the high amount of accumulated pulcherrimin in the yeast cells (Figure 2, Table 3). Similar observations were reported previously by Sipiczki [20,42], where the intensity of colony pigmentation increased with the Fe^{3+} concentration. Both the widest "pulcherrimin halos" and the darkest colony colour were observed for *M. pulcherrima* TK1, which proves the highest production of pulcherriminic acid by this strain among other tested, and, in consequence, the highest potential as a biocontrol agent for potato protection. This observation is in convergence with the antimicrobial in vitro test (Figure 1).

Table 3. Pulcherrimin formation for selected *Metschnikowia pulcherrima* strains on YPD medium supplemented with various concentrations of $FeCl_3$.

$FeCl_3$ Supplementation (mg/mL)	Growth Features and Pulcherrimin Formation	Strain				
		J2	J3	J6	TK1	M4
Without (control)	Colony colour top/bottom	white/creamy	white/creamy	white/creamy	white/creamy	white/creamy
	Coloured halo (mm)	0.5	0.5	0.5	4.0	0.0
0.005	Colony colour top/bottom	white/creamy	white/creamy	white/creamy	white/creamy	white/creamy
	Coloured halo (mm)	1.0	1.0	2.0	6.0	1.0
0.01	Colony colour top/bottom	white/red	white/red	white/red	white/red	white/red
	Coloured halo (mm)	0.5	0.0	0.0	1.5	0.0
0.2	Colony colour top/bottom	red/red	red/red	red/red	red/dark red	red/red
	Coloured halo (mm)	0.0	0.0	0.0	0.0	0.0

J2—M. pulcherrima J2; J3—M. pulcherrima J3; J6—M. pulcherrima J6, TK1—M. pulcherrima TK1; M4—M. pulcherrima M4.

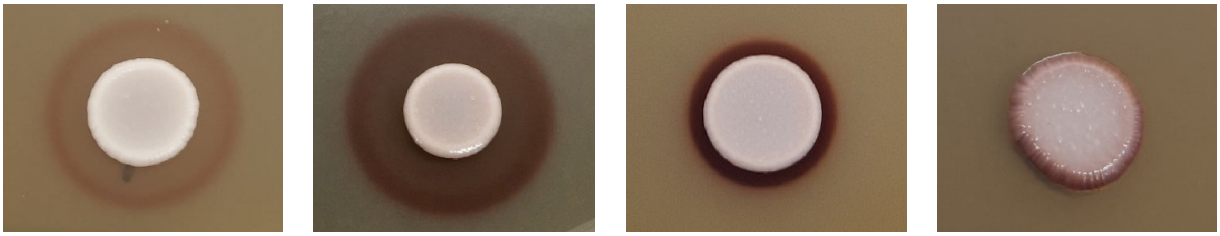

Figure 2. Halo formation and intensity of colony pigmentation of *Metschnikowia pulcherrima* TK1 cultured on YPD agar. (**A)**—without $FeCl_3$, supplemented with (**B)**—0.005 mg/mL $FeCl_3$, (**C)**—0.01 mg/mL $FeCl_3$, and (**D)**—0.2 mg/mL $FeCl_3$.

Iron sequestration is not the only antimicrobial mechanism of *M. pulcherrima* yeasts. The secretion of enzymes that degrade cell walls, cell membranes, and other cellular components is often highlighted as the mode of action of yeast biocontrol [19,20]. The enzymatic activity of selected strains of *Metschnikowia* yeasts was assessed using the API ZYM test. The results are presented in the heat map (Figure 3). All tested strains exhibited enzymatic activity of α- and β-glucosidase, acid phosphatase, cystine, valine and leucine arylamidase, esterase lipase (C8), and esterase (C4). The highest activity was observed for leucine arylamidase. This enzyme is involved in the production of leucine, which is needed for cyclodileucine (cyclo(Leu-Leu)) formation—the precursor of pulcherriminic acid [43]. The level of α- and β-glucosidase activity was marked as low to high, depending on the yeast strain. These enzymes interfere with the fungal cell membranes [44] and cause the subsequent death of the pathogen. The highest enzymatic potential was observed for the *M. pulcherrima* TK1 strain. Similar enzymatic profiles were also noted for other strains of the *M. pulcherrima* clade isolated from Polish fruits and flowers [18,45].

In addition, the production of organic acids, ethanol, and glycerol can contribute to the overall antifungal effect of *Metschnikowia* yeasts [46]. Therefore, we also checked the metabolic profiles of selected yeast isolates (Figure 4). In HPLC analysis, ethanol was identified as the main fermentation product of *Metschnikowia* yeasts cultivated in YPD broth (Figure 4). The highest value was obtained for two strains: *M. pulcherrima* TK1 (835.27 mg/100 mL) and *M. pulcherrima* J2 (837.68 mg/100 mL). In addition to ethanol, HPLC analysis determined smaller amounts (<80 mg/100 mL) of glycerol and organic acids such as succinic, lactic, and acetic acid in post-culture YPD media. Glycerol was the second-most-abundant metabolite, ranging from 40.58 to 76.41 mg/100 mL for the J3 and TK1 strains, respectively. On average, the succinic acid level reached about 6.5 ± 1.8 mg/100 mL. The lactic acid concentration ranged from 11.28 to 45.71 mg/100 mL for the J2 and J6 strains, respectively, and the amount of acetic acid ranged from 2.16 to 24.67 mg/100 mL for the J3 and J2 strains, respectively (Figure 4). Acetic acid was reported previously as the main antifungal component, which was more effective than lactic acid [47]. With the effect of the external chemical alteration of and change in pH, the structure of cell membrane units such as proteins and phospholipids are altered. These alterations also affect the permeability of the cell membrane and may result in leakage of the internal cell metabolites. Additionally, acids penetrate the fungal cell and adversely affect intracellular activities such as DNA replication and protein synthesis [48].

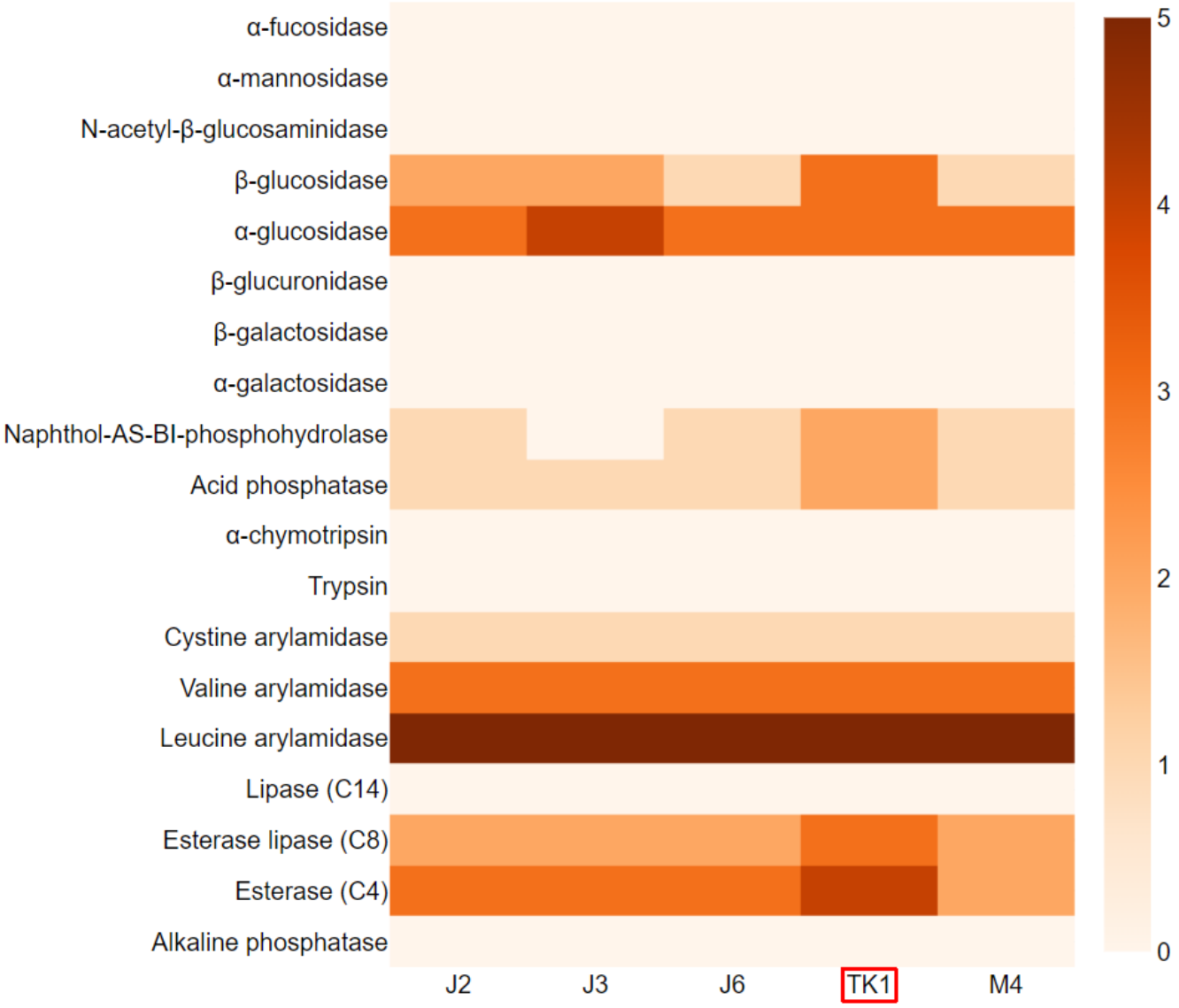

Figure 3. Enzymatic activity of the selected strains of *Metschnikowia pulcherrima* strains based on API ZYM system. 0—no enzyme activity; 5—the highest level of enzymatic activity; J2—*M. pulcherrima* J2; J3—*M. pulcherrima* J3; J6—*M. pulcherrima* J6; TK1—*M. pulcherrima* TK1; M4—*M. pulcherrima* M4.

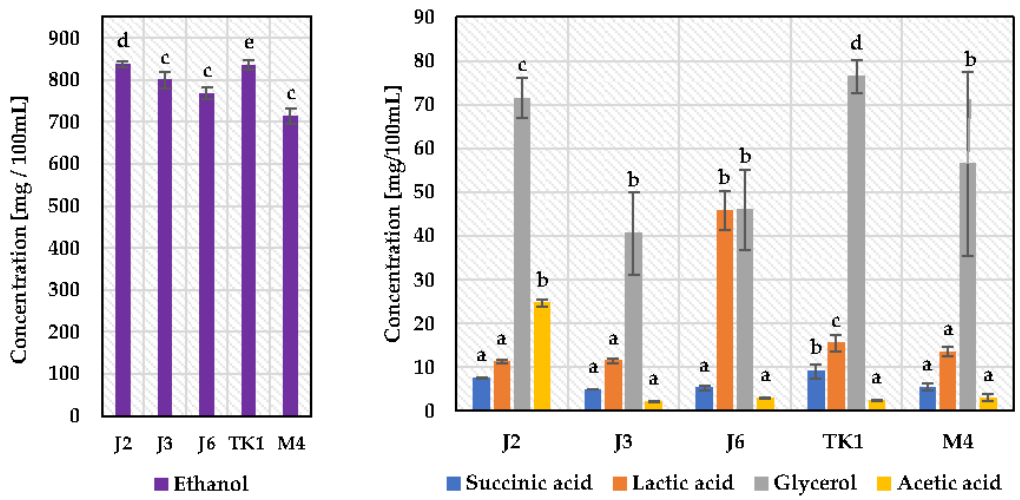

Figure 4. Fermentation products of the most active *Metschnikowia pulcherrima* strains cultivated on YPD medium. J2—*M. pulcherrima* J2; J3—*M. pulcherrima* J3; J6—*M. pulcherrima* J6; TK1—*M.*

pulcherrima TK1; M4—*M. pulcherrima* M4. a–e results, with different letters within the sample, are significantly different (Tukey's test, $\alpha = 0.05$).

M. pulcherrima TK1 was selected for further research, as the most active strain with the highest biocontrol potential based on its antimicrobial and enzymatic properties and metabolite profile.

3.3. Growth Medium Selection for M. pulcherrima TK1 Cultivation

In our work, acid whey was enriched with nitrogen sources (yeast extract and peptone) in 16 different concentration variants and with glucose as an additional carbon source in 27 other variants (including yeast extract and peptone). The naturally occurring lactose in the acid whey was the basic carbon source. The results of maximum specific growth rate (μ_{max}) and biomass yield (Y_x) are presented in Figure 5.

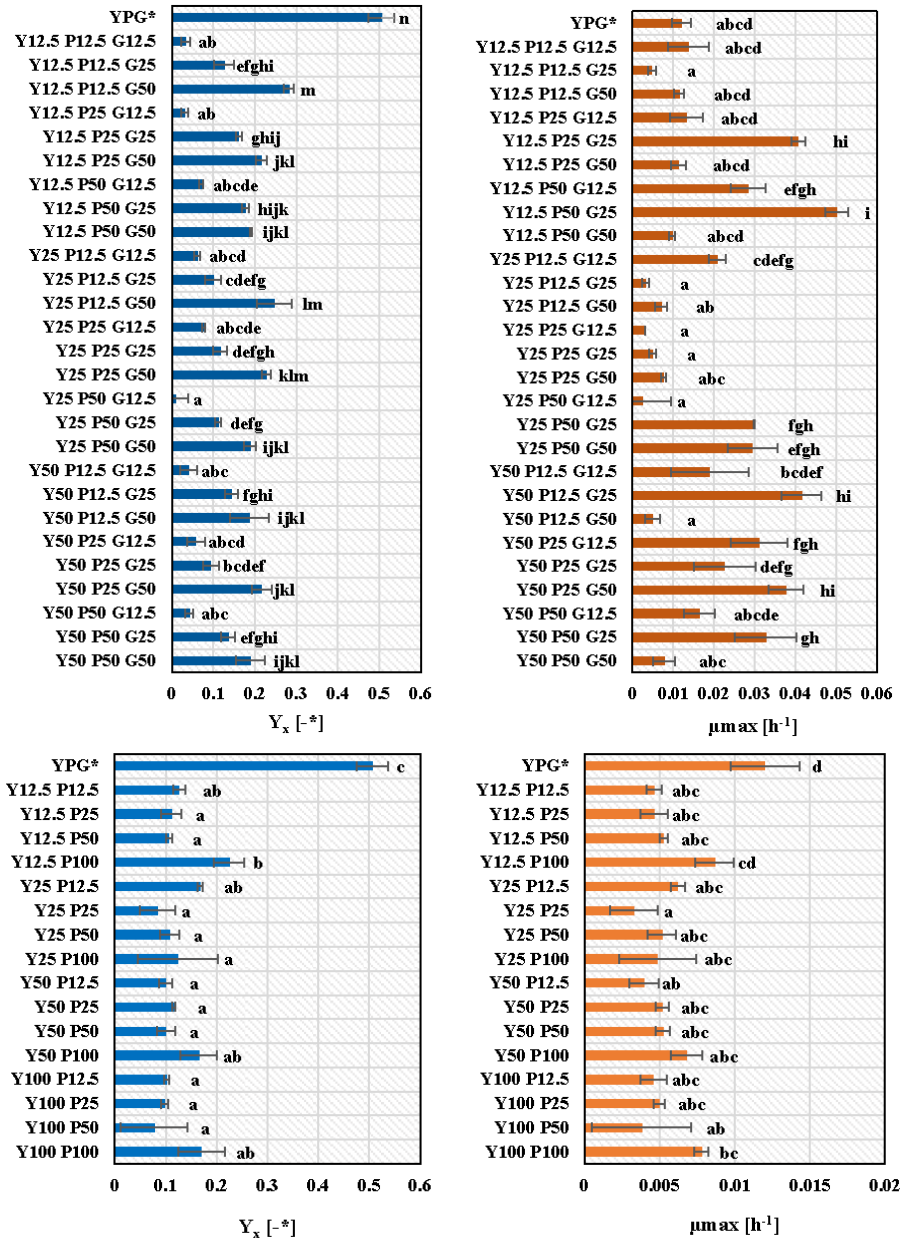

Figure 5. Biomass yield (Y_x) and maximum specific growth rate (μ_{max}) for *Metschnikowia pulcherrima* TK1 strain cultivated in different variants of acid-whey-based broth with glucose supplementation (YPG samples) and without glucose supplementation (YP samples). * laboratory broth, water-based;

Y—yeast extract; P—peptone; G—glucose; Y100 P100—acid-whey-based medium supplemented with base 10 g/L of yeast extract and 20 g/L of peptone; Y50 P50 G50—acid-whey-based medium supplemented with base 5 g/L concentration of yeast extract, 10 g/L of peptone, and 10 g/L of glucose. The following numbers indicate supplementation with a certain percentage of the base concentration of Y, P, and G. The specific concentrations of each component are presented in 2.7. of Materials and Methodology section. a–m results, with different letters within the sample, are significantly different (Tukey's test, $\alpha = 0.05$).

Among the variants with glucose supplementation, the highest level of biomass yield was achieved for media Y25 P25 G50, Y25 P12.5 G50, and Y12.5 P12.5 G50, ranging from 0.2286 to 0.2827 (Figure 5). The biomass yield for variants without glucose enrichment was lower. The three variants with the highest biomass yield ranged between 0.1680–0.2248 (Y12.5 P100, Y100 P100, Y25 P12.5). In the samples supplemented only with the yeast extract and peptone, the peptone concentration was more significant in achieving a satisfactory level of Y_x than the yeast extract. Glucose concentration was critical in the other samples. Overall, the highest Y_x was obtained in the model YPG medium, which is related to the previous adaptation of *M. pulcherrima* TK1 to these conditions (Figure 5).

M. pulcherrima TK1 cultures on six variants of the acid-whey-based medium (three with glucose, three without) with the highest Y_x were selected for further antimicrobial activity assessment against potato phytopathogens. The results are presented in Figure 6.

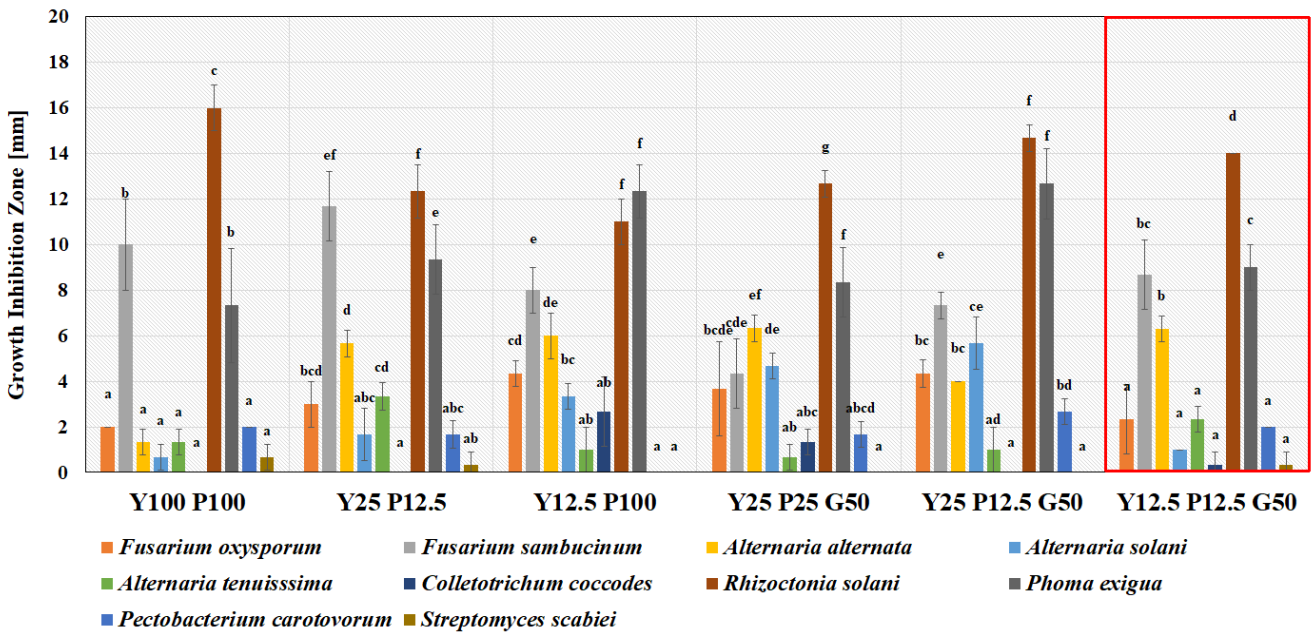

Figure 6. Antimicrobial activity of *Metschnikowia pulcherrima* TK1 strain cultivated on different variants of acid-whey-based media against potato phytopathogens measured as growth-inhibition zones in the agar-well diffusion method. Y100 P100—acid-whey-based medium supplemented with base 10 g/L of yeast extract and 20 g/L of peptone; Y50 P50 G50—acid-whey-based medium supplemented with base 5 g/L concentration of yeast extract, 10 g/L of peptone, and 10 g/L of glucose. The following numbers (50, 25, 12.5) indicate supplementation with a certain percentage of the initial concentration of yeast extract, peptone, and glucose. The specific concentrations of each component are presented in 2.7. of Materials and Methodology section. a–f results, with different letters within the sample, are significantly different (Tukey's test, $\alpha = 0.05$).

Interestingly, the diversified spectrum of antimicrobial activity varied and depended on the kind of culture medium (Figure 6). The widest spectrum of activity was noted for *M. pulcherrima* TK1 cultivated in the broth Y12.5 P12.5 G50, with the lowest concentration of supplements among glucose variants. Tested strain TK1, after multiplication in this broth, inhibited the growth of all tested potato phytopathogens. The inhibition zones

ranged from 0.3 mm (for *S. scabiei* and *C. coccodes*) to 14.0 mm (for *Rhizoctonia solani*). The most susceptible pathogens were *R. solani*, *P. exigua*, and *F. sambucinum* (Figure 6). Convergent results were observed for *R. solani* and *F. sambucinum* in experiments with the YPG culture; however, *P. exigua* was previously the most resistance strain (Figure 1). In this antimicrobial test, *C. coccodes* exhibited the highest resistance; it was inhibited by only three variants of the supplemented acid-whey-based cultures of selected yeasts. It is worth noting that the growth of both bacterial potato pathogens (*P. carotovorum* and *S. scabiei*) was inhibited by several variants of acid whey cultures of *M. pulcherrima* (Figure 6). At the same time, no antibacterial activity was observed for the strain cultivated in the YPG medium (Figure 1).

The acid-whey-based medium Y12.5 P12.5 G50 was finally selected for further cultivation of *M. pulcherrima* TK1 due to its broadest spectrum of antimicrobial activity and high level of μ_{max} and Y_x. The use of acid whey as a base for the culture medium allowed for an eightfold reduction in the concentration of peptone and yeast extracts and a twofold reduction in glucose concentration, while maintaining activity against mould pathogens and even the appearance of activity against bacterial pathogens.

Determination of the Y12.5 P12.5 G50 acid-whey-based medium chemical characteristic was performed. The results are presented in Table 4. The selected variant of culture medium contained higher concentrations of nitrogen (75.76 mg/L), iron (28.00 mg/L), potassium (104.50 mg/L), and calcium (289.00 mg/L). Lactose (9 g/L) from acid whey was the second carbon source, in addition to glucose.

Table 4. Characteristic of acid-whey-based medium selected for the cultivation of *Metschnikowia pulcherrima* TK1.

Analytical Analysis	Medium		
	Acid Whey Broth Supplemented Y12.5 P12.5 G50	Acid Whey Broth without Supplementation	YPG Broth
Phosphorus (mg/L)	175.56	181.90	182.08
Nitrogen (mg/L)	75.76	38.20	62.59
Iron (mg/L)	28.00	0.50	5.00
Potassium (mg/L)	104.5	75.00	65.70
Calcium (mg/L)	289.00	324.55	9.20
Chloride (mg/L)	32.80	57.10	0.18
Sodium (mg/L)	38.90	62.85	0.26
Lactose (g/L)	9.00	14.55	0.20
Protein (g/kg)	11.38	8.50	32.64
Lactic acid (g/L)	16.30	15.85	1.22
Total solids (g/kg)	78.71	58.00	58.18
Volatile solids (g/kg)	69.37	48.05	55.04
Water content (g/kg)	921.29	941.10	941.82

The *M. pulcherrima* TK1 strain cultivated in the selected Y12.5 P12.5 G50 acid-whey-based medium showed slightly modified metabolite profiles, in favour of lactic acid and acetic acid production, and lowered the concentration of glycerol, ethanol, and succinic acid (Figures 7 and 8). Probably over 28 higher concentrations of lactic acid in A100 M100 acid-whey-based post-culture medium of TK1 compared to YPD were caused mainly by the natural presence of this acid in whey.

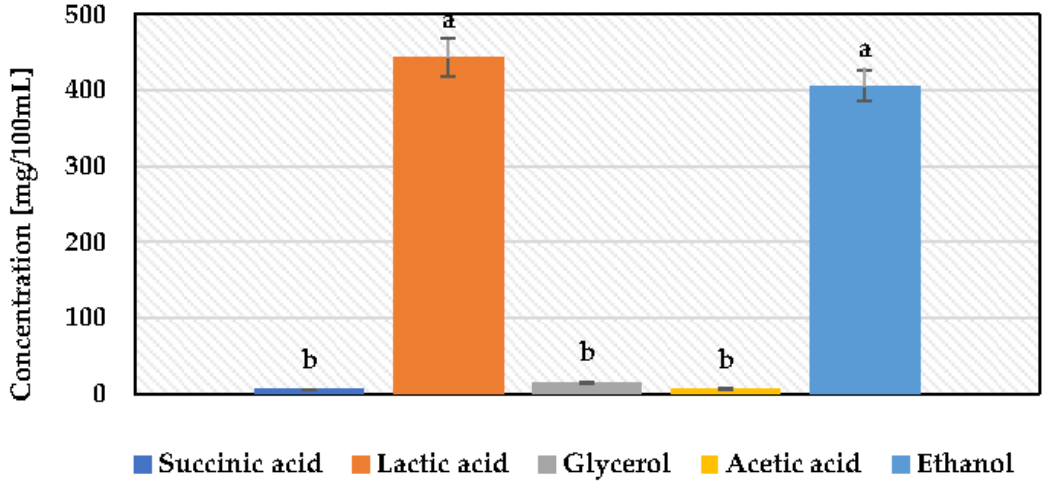

Figure 7. Fermentation by-products of the *M. pulcherrima* TK1 cultured on Y12.5 P12.5 G50 acid-whey-based medium.

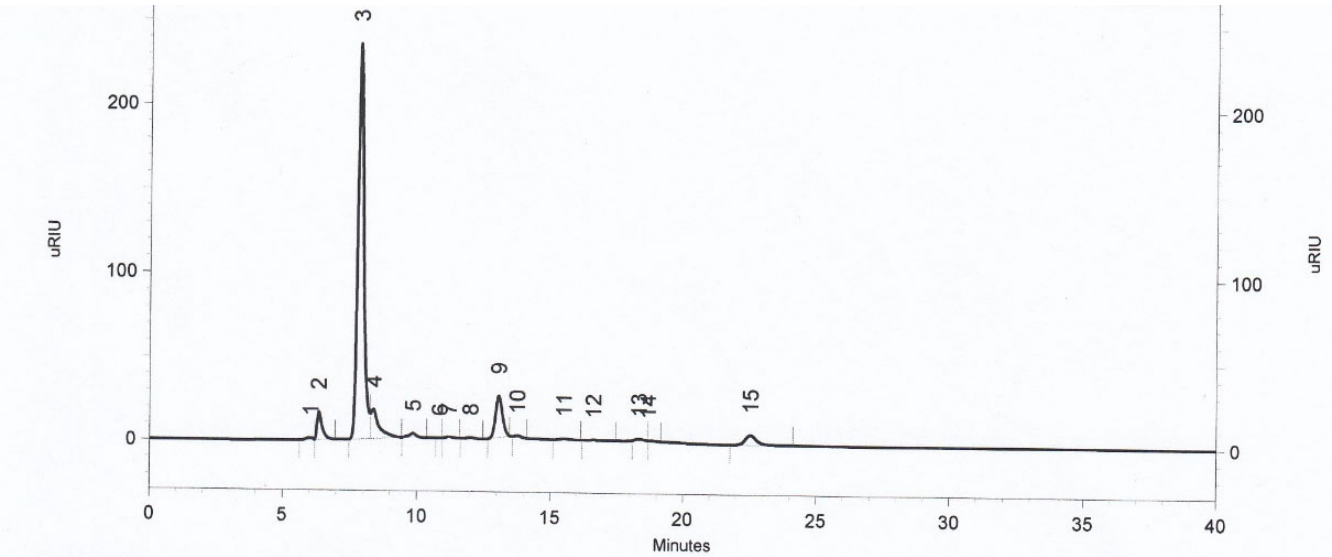

Figure 8. RI chromatogram of *M. pulcherrima* TK1 cultured on Y12.5 P12.5 G50 acid-whey-based medium; 8—succinic acid; 9—lactic acid; 10—glycerol; 11—acetic acid; 15—ethanol.

3.4. In Situ Test of Biocontrol Potential of M. pulcherrima TK1 against Phytopathogens on Potatoes

The in situ antimicrobial activities of *M. pulcherrima TK1* cultured in the acid-whey-based broth Y12.5 P12.5 G50 and reference YPG medium against the 10 phytopathogens were evaluated on 'Impresja' seed potatoes. The results are presented in Table 5 and Figure 9.

Table 5. Inhibition of potato seed cv. *Impresja* infestation treated with *Metschnikowia pulcherrima* TK1 cultured on Y12.5 P12.5 G50 acid-whey-based medium and YPD against potato phytopathogens.

Phytopathogens	Inhibition of Potato Infestation (%)	
	Acid Whey Broth Supplemented Y12.5 P12.5 G50	YPD Broth
Fusarium oxysporum	30 ± 10	30 ± 5
Fusarium sambucinum	100 ± 0 *	80 ± 10 *
Alternaria alternata	100 ± 0 *	80 ± 5 *

Alternaria solani	100 ± 0 *	90 ± 5 *
Alternaria tenuissima	100 ± 0	95 ± 5
Colletotrichum coccodes	100 ± 0	100 ± 0
Rhizoctonia solani	50 ± 5	60 ± 5
Phoma exigua	100 ± 0 *	80 ± 5 *
Pectobacterium carotovorum	-	-
Streptomyces scabiei	40 ± 10 *	20 ± 5 *

- no pathogen inhibition; * statistically significant differences between YPG medium and Y12.5 P12.5 G50 acid-whey-based medium for each pathogen (one-way ANOVA, $\alpha < 0.05$).

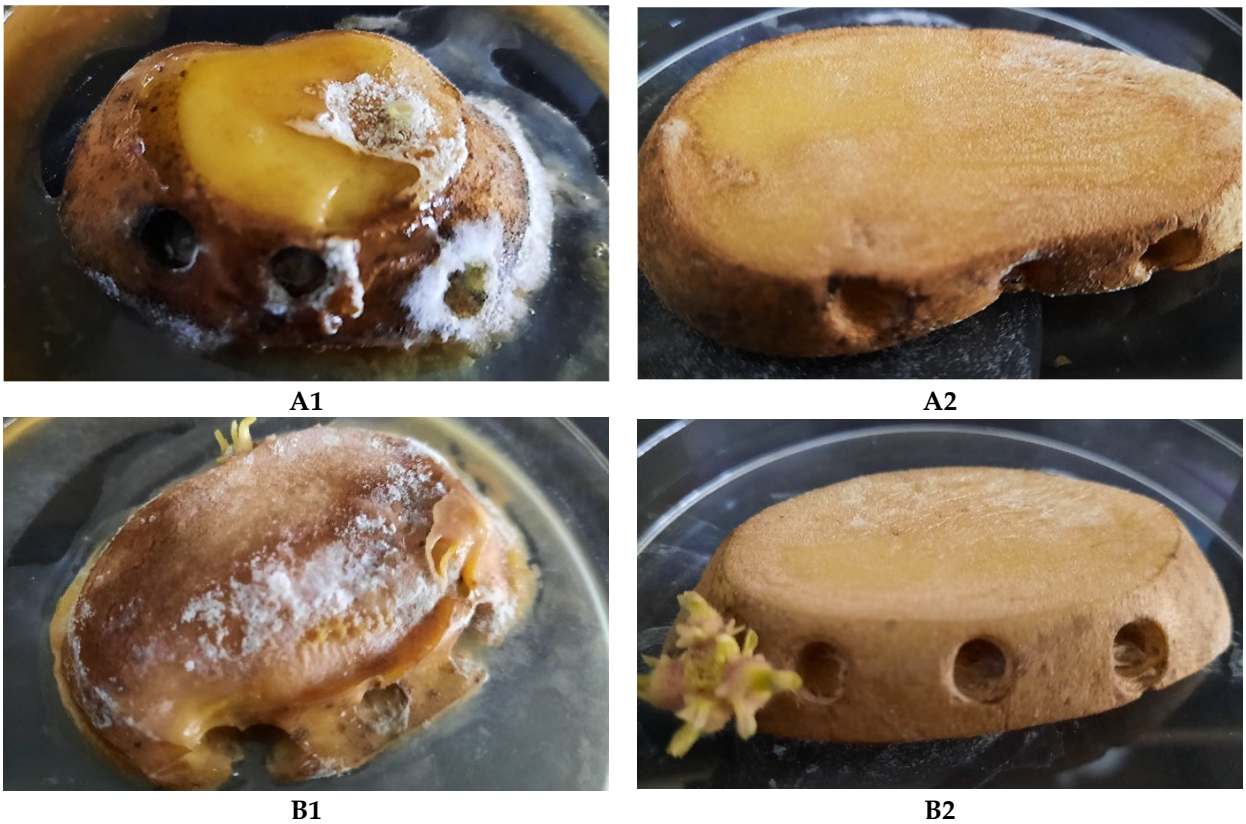

Figure 9. Inhibition of *Alternaria solani* (A) and *Fusarium sambucinum* (B) infestation of potatoes cv. Impresja treated with *Metschnikowia pulcherrima* TK1 cultured in the acid-whey-based medium Y12.5 P12.5 G50. (**A1**,**B1)**—control samples; (**A2**,**B2)**—tested samples.

Several studies reported the biocontrol efficiency of *Metschnikowia* yeast in situ against brown rot in sweet cherries [49], anthracnose in mango fruit [50], and grey mould in strawberries [18] and grape berries [35]. To the best of our knowledge, this is the first work that confirms the antimicrobial activities of *M. pulcherrima* against *F. oxysporum*, *F. sambucinum*, *P. exigua*, *C. coccodes*, *R. solani*, *A. solani*, *A. alternata*, *A. tenuissima*, and *S. scabiei* in situ on potatoes. It was observed that *M. pulcherrima* TK1 cultured on Y12.5 P12.5 G50 acid-whey-based medium completely (100% inhibition) or partially (30%–50% inhibition) reduced the potato infestation, with all of the tested fungal pathogens and one bacterial pathogen (Table 5, Figure 9). Only the development of *P. carotovorum* was not reduced, even though the in vitro experiment revealed the inhibition of this bacterium by *M. pulcherrima* TK1 culture in the Y12.5 P12.5 G50 variant (Figure 6). Cultivation of *M. pulcherrima* TK1 on enriched acid-whey-based medium achieved a higher or at least the same percentage of potato infestation as the YPG model medium. The most effective inhibition (100%) was achieved for all three *Alternaria* strains, *F. sambucinum*, *C. coccodes*, and *P. exigua*. Guo et al. study [38] also showed a positive effect of treatment with *M. pulcherrima* against *A. alternata* on winter jujube fruit after seven days of storage. However, the

antimicrobial effect was observed to a lesser extent (26%). The least satisfactory level of inhibition was noted in our work for *F. oxysporum* (30%). In Türkel et al.'s research [23], *Fusarium* sp. growth was completely inhibited when co-inoculated with *M. pulcherrima* into artificially wounded apples.

Application of *M. pulcherrima* TK1 treatment was allowed to inhibit all tested fungal phytopathogens of potato. *P. carotovorum* (Gram-negative bacteria) was insensitive to yeast treatment in situ and *S. scabiei* (Gram-positive bacteria) was 40% inhibited. Therefore, it is essential to select a microorganism or other molecule that is active against fungi or bacteria (Gram-negative or Gram-positive). Biological control agent should exhibit a high specificity of action to be an alternative for chemical pesticides. Our previous research [29] on potato protection using lactic acid bacteria as a biocontrol agent confirmed the antibacterial activity of *Lactiplantibacillus plantarum* KB2 LAB 03 against *P. carotovorum* and *S. scabiei* at a satisfactory level (90% and 85% growth reduction, respectively). Additionally, the garlic water extract completely inhibited the growth of *S. scabiei* development (growth reduction by 100%) but was ineffective against *P. carotovorum* [28]. The possible synergistic effect of these biopreparations could cover the need for comprehensive protection of potatoes against fungal and bacterial pathogens. However, more studies on mutual interactions are required.

4. Conclusions

The results from this study clearly showed that the selected isolates of *M. pulcherrima* could effectively control the diseases of potatoes mainly caused by fungal phytopathogens. The modes of potential mechanisms may include not only the iron depletion but also the enzymatic action and organic acid production of the active biocontrol agent. Interestingly, the metabolic profiles of *M. pulcherrima* can also be modulated by using an appropriate growth medium. The acid-whey-based broth with little supplementation was a cheap medium suitable for the multiplication of *M. pulcherrima* cells with strong biocontrol properties. The obtained results provided an impulse for further research involving the use of yeasts during the storage of potato seeds in cooling chambers. Such a strategy, based on combining the use of a selected biological agent and appropriately designated physicochemical parameters of the chamber, seems to be the key solution for significantly reducing losses during the storage of potato seeds.

5. Patents

No. P.441895: Method of bio-protection of seed potato tubers against the development of fungal phytopathogens, patent application at the patent office in Poland, 1 August 2022.

Author Contributions: Conceptualisation, A.S., J.B., D.K., and B.G.; formal analysis, A.S., A.C., and M.O.; funding acquisition, B.G.; investigation, A.S., A.K., A.C., J.S., and W.C.-W.; methodology, A.S., J.B., and J.S.; project administration, B.G.; supervision, D.K. and B.G.; visualisation, A.S.; writing—original draft, A.S. and A.K.; writing—review and editing, D.K. and B.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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