Application of surface-active substances produced by *Rhodococcus erythropolis* IMB Ac-5017 for post-harvest treatment of sweet cherry

Tetiana Pirog[1,2], Viktor Stabnikov[1], Svitlana Antoniuk[1]

1 – National University of Food Technologies, Kyiv, Ukraine
2 – Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kyiv, Ukraine

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Corresponding author:

Viktor Stabnikov
E-mail: vstabnikov1@gmail.com

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Abstract

Introduction. The aim of the present study was testing of the supernatant of *Rhodococcus erythropolis* IMB Ac-5017 with different concentration of surface-active substances (SAS) for treatment of sweet cherry for shelf-life extension.

Materials and methods. *R. erythropolis* IMB Ac-5017 were grown in the medium with ethanol. Supernatant with concentration of SAS from 0.1 to 0.5 g/L was used for the treatment of sweet cherry fruit. Concentration of SAS in supernatant was determined by weight method. The total number of heterotrophic bacteria and fungi were determined by the plate dilution method.

Results and discussion. The treatment of sweet cherries with a supernatant containing 0.5 g/L SAS diminished the numbers of bacteria and fungi on the fruit's surface by 10 and 5 times, respectively, in comparison with cherries washed with water. The treatment of sweet cherries with supernatant containing 0.2 g/L SAS diminished the numbers of bacteria and fungi on the fruit's surface by 5 and 3 times, respectively; treatment with supernatant containing 0.1 g/L diminished the numbers of bacteria and fungi by 2 times in comparison with cherries washed with water. The treatment with supernatant with concentration SAS 0.5 g/L was most effective. Treated with supernatant sweet cherries fruits did not show signs of decay even on 7th day of storage, while untreated or washed with water fruits lost moisture, fruit's skin became wrinkled, cracks and decayed areas appeared on it.

Content of fungal cells on the surface of sweet cherry pretreated with supernatant with concentration of SAS from 0.1 to 0.5 g/L and after that contaminated with spore's suspension of *Aspergillus niger* P-3 were by 2 – 11 times lower than on the surface of fruits washed with water after 5 days of incubation.

The possibility of multiple usage of supernatant was shown. Application of supernatant with concentration of 0.5 g/L resulted in decrease of bacterial concentration after first usage by 10 times, after second usage it was diminished by 5 times and after third usage it was diminished by 3 times, meanwhile concentration of fungi decreased by 9, 5 and 4 times after I, II, and III usage of supernatant.

Conclusion. Surface-active substances synthesized by *Rhodococcus erythropolis* IMB Ac-5017 could be used for treatment of sweet cherry to extend their shelf life.

Introduction

The harvest season of sweet cherries is short; these fruits are extremely perishable and spoil easily after harvest due to physical damage during harvesting, transportation, water loss during the storage and rapid microbiological deterioration of the stored fruits. Therefore, even a short extension of shelf life due to postharvest treatments will be profitable for the fresh sweet cherries market. Different methods for sweet cherry fruit preservation have been developed. Traditional methods for maintenance of fresh-cut cherry quality include regulation of temperature and humidity (Chockchaisawasdee et al., 2016). Treatments of different fruits and vegetables with chemicals are widely used to prolong their postharvest storage life. To coat fruits and vegetables with chemicals, immersion or sprinklings are usually used (Golding, 2017; Suslow, 2005). Among the chemicals, chlorination is effective and relatively inexpensive method for reduction of the incidence of postharvest diseases. To decrease the quantity of microbial cells on the surface of fruits and vegetables, they can be immersed in water with added chlorine-containing substances – salts (calcium hypochlorite or sodium hypochlorite) or gases (chlorine gas or chlorine dioxide). It is known that this method is applied for the treatment of sweet cherries, melons, apples, pears, tomatoes, peppers, potatoes, and salads (Suslow, 2005). Treatment with fungicides is also provided by immersion of fruits or vegetables in their solutions, but if the amount of harvested fruits or vegetables is not too big, a sprinkling is used (Golding, 2017). In spite of the effectiveness of chemicals, their application is not appreciated by consumers because of health concerns. So, alternative safe methods have to be developed. Different biological methods for post harvested treatment of fruits are intensively studied. They include application of edible coating made of natural polysaccharide chitosan (Pasquariello et al., 2015; Romanazzi et al., 2018), natural biocides such as plant essential oils and methyl jasmonate (Maghenzani et al., 2018), microbial antagonists (Dukare et al., 2019; Lastochkina et al., 2019), and also combination of different biological agents (Guo et al., 2014; de Oliveira et al., 2017).

In recent years, several studies have been carried out to establish the possibility of biosurfactants – microbial surface-active substances (SAS) – application to extend shelf-life for fresh-cut fruits (Adetunji et al., 2018; Toral et al., 2018). The aim of the present study was testing of the supernatant of *Rhodococcus erythropoli*s IMB Ac-5017 with different concentration of SAS for the treatment of sweet cherry for shelf-life extension.

Materials and methods

Microorganisms

The strain *Rhodococcus erythropolis* was isolated from the oil-polluted soil and was deposited in the Collection of Microorganisms of Institute of Microbiology and Virology, National Academy of Science, Ukraine as *Rhodococcus erythropolis* IMB Ac-5017 (Pirog et al., 2020). This strain produces extracellular surface-active substances, which contain glycolipids (trehalose mono- and di-mycolates), neutral lipids and phospholipids (Pirog et al., 2013).

To determine antifungal activity of surface-active substances produced by *R. erythropolis* IMB Ac-5017, the fungal strain *Aspergillus niger* P-3 from the Collection of Microorganisms of the Department of Biotechnology and Microbiology, National University of Food Technologies, Ukraine, was used as a test culture.

Cultivation of *R. erythropolis* IMB Ac-5017

The liquid mineral medium with the following composition, g/L: $NaNO_3$, 1.3; $MgSO_4 \cdot 7H_2O$, 0.1; NaCl, 0.1; Na_2HPO_4, 0.16; KH_2PO_4, 0.14; $CaCl_2$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.001; distilled water up to 1L, pH 6.8–7.0 was used for cultivation of the bacterial strain *R. erythropolis* IMV Ac-5017. Ethanol, 2% (v/v), was a source of the carbon and energy.

Inoculum was produced by the cultivation of bacterial strain in the liquid mineral medium of the same composition as shown above with 0.5% (v/v) of ethanol. Inoculum with the concentration of the cells of 10^4–10^5 cells/mL was taken from the exponential phase of growth and added to the medium for *R. erythropolis* IMV Ac-5017 cultivation in quantity of 10% (v/v).

Cultivation of *R. erythropolis* IMB Ac-5017 was conducted in the 750 mL flasks with the 100 mL of medium under shaking 320 rpm at 30 ºC during 120 hours.

Determination of surface-active substances concentration

The amount of surface-active substances (SAS) synthesized by *R. erythropolis* IMB Ac-5017 was determined by weight method. The culture liquid was centrifuged at 5000×g for 45 minutes (laboratory centrifuge LP–8, Kiev, Ukraine). The Folch solution (chloroform and methanol in volume ratio 2:1) was used for extraction of surface-active substances as it was described earlier (Pirog et al., 2019).

Preparation of SAS-containing supernatant

The cultural liquid after cultivation of *R. erythropoli*s IMB Ac-5017 was centrifuged at 5000×g for 25 minutes. Supernatant was separated from bacterial biomass and sterilized for 30 min at 112 °C. Fruits were treated with supernatant with concentration of SAS from 0.1 to 0.5 g/L. To achieve the desired concentration, supernatant was diluted by the addition of sterile tap water.

Fruits treatment

Fruits of the sweet cherry cultivar “Regina” were picked by hand from the trees cultivated without pesticides in the Experimental station, Gvozdev, Kyiv Oblast, Ukraine, GPS 50°14'53.5"N 30°28'41.3"E. The harvested fruits were ripe, without visible damages and infections. Selected fruits were divided into three groups with 10 – 30 pieces in each. The fruits from the first group were not treated at all, fruits of the second group were washed with tap water, and fruits of the third group were washed with supernatants with concentration of SAS from 0.1 to 0.5 g/L.

Fruits from second and third groups were placed in the glass cylinder, 250 mL of tap water or supernatant was added, treatment lasted for 5 min, and after that fruits were taken off and supernatant was reused to treat new group of fruits. The procedure was repeated and the third group of fruits was treated with the same supernatant. So, one solution of supernatant was used to tread three different groups of fruits. Untreated and treated fruits were placed on the plates and left at room temperature for observation. Microbiological analysis was done before the beginning of the fruit’s storage.

Microbiological analysis

Some fruits from each group were taken aseptically and then were homogenized for 3 min using dispersing instrument T 10 basic ULTRA-TURRAX. Homogenized mixture, 1 g, was placed in the tube with 9 mL of sterile water and was shaken vigorously. The quantity of microbial cells (colony-forming units, CFU) was determined by the plate dilution method. The quantity of heterotrophic bacteria was determined by their growth on the meat-and-peptone agar at 30 ºC for 24 hours, and the quantity of fungi was determined by their growth on the wort agar-agar at 24ºC for 48 hours.

Evaluation of antifungal activity of SAS containing supernatant of *R. erythropolis* IMB Ac-5017 against fungi *Aspergillus niger* P-3

Antifungal activity of SAS containing supernatant of *R. erythropolis* IMB Ac-5017 was determined by the following method described in (Matei e al., 2016). Selected fruits were divided into three groups with 10 – 30 pieces. Half of the fruits were bruised with a sterile lancet, and then fruits were washed with water or supernatant with different SAS concentration as it was described above. After 30 min, all sweet cherry fruits were sprayed with spore suspension (10^6 spores/mL) of fungi *Aspergillus niger*, which is one of the most common infectious agents of post-harvest spoilage of sweet cherry. After incubation, some sweet cherries from each group were taken with sterile pincette, were homogenized and microbiological analysis were performed.

Evaluation of fruits quality

Evaluation of sweet cherry fruits quality was done by viewing during the storage time. The experiment was finished when the signs of deterioration (usually on the seventh day) such as decay, changes of color and texture, the presence of the cracks and wrinkling were evident on all fruits.

Statistical analysis

The experiments were carried out in triplicates and the number of the parallel determinations varied from 3 to 5. Statistical analysis was done using computer program Statistix 10.0 for Windows version 11.5. The average means and standard deviations were calculated for the experimental results.

Results and discussion

Effect of concentration of SAS in supernatant of *R. erythropolis* IMB Ac-5017 and method for the treatment of sweet cherries on numbers of heterotrophic bacteria and fungi on the fruit's surface

Quality of fresh-cut sweet cherries are usually evaluated by their appearance, texture, colors, firmness, chemical composition, level of physiological activity and microbial characteristics such as a percent of fungal infections and the level of mesophilic bacteria on the surface of fruits (Asghari, 2019; Maghenzani 2018). Microbial load on the surface of

fresh-picked vegetables is not obviously negatively correlated with time of their storage, but this linkage becomes critical in case of fruits storage (Fan and Song, 2008). This is associated with high content of sugar in fruits which may cause rapid microbial spoilage. In comparison with cherries which have high content of organic acids, 1.5–1.8%, and 8−20% of sugar, content of sugars in sweet cherries is higher, 13–25% , and organic acids significantly lower, 0.4–1.5% (Chockchaisawasdee et al., 2016) that is one of the reasons of their susceptibility to microbial spoilage.

Decrease of microbial contamination on the surface of sweet cherries could increase the time of their storage, so first step in our investigation was determination of influence of the treatment of sweet cherries with supernatant with different concentration of SAS on the microbial numbers on the fruits surface (Fig. 1).

The numbers of bacteria and fungi on the surface of fruits washed with water were $5.4 \cdot 10^3$ colony forming units (CFU)/mL and $2 \cdot 10^3$ CFU/mL, respectively.

Treatment of sweet cherries with a supernatant containing 0.5 g/L SAS diminished the numbers of bacteria and fungi on the fruit's surface by 10 and 5 times, respectively, in comparison with cherries washed with water. The treatment of sweet cherries with supernatant containing 0.2 g/L SAS diminished the numbers of bacteria and fungi on the fruit's surface by 5 and 3 times, respectively; treatment with supernatant containing 0.1 g/L diminished the numbers of bacteria and fungi by 2 times in comparison with cherries washed with water. The treatment with supernatant with concentration SAS 0.5 g/L was most effective (Figure 1). Treated with supernatant sweet cherries fruits did not show signs of decay even on 7th day of storage, while untreated or washed with water fruits lost moisture, fruit's skin became wrinkled, cracks and decayed areas appeared on it (Figure 2).

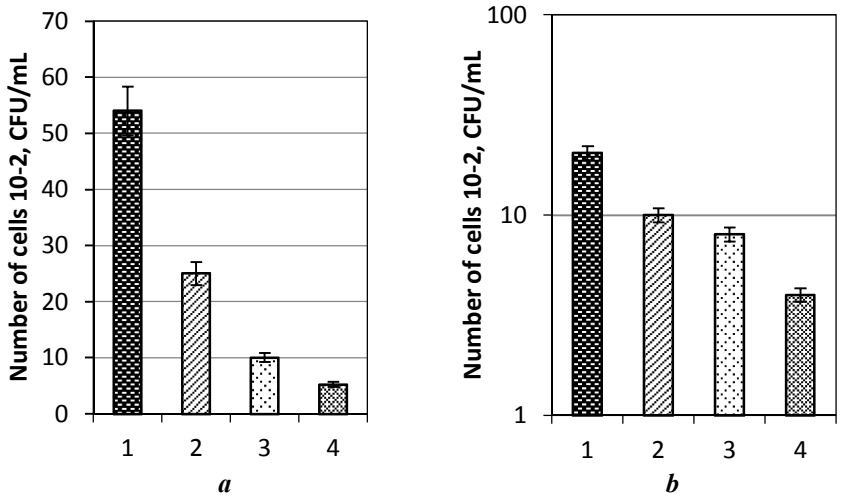


Figure 1. The total number of heterotrophic bacteria (A) and fungi (B) depends on the method of sweet cherries treatment: washing with water (1); the treatment with supernatant of *R. erythropolis* IMB Ac-5017 with SAS concentration: 0.1 g/L (2); 0.2 g/L (3); 0.5 g/L (4).

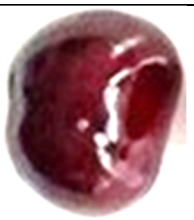
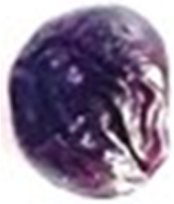
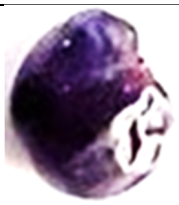
On day		1st	7th
Control			
Washing with water			
SAS concentration, g/L	0.1		
	0.2		
	0.5		

Figure 2. Effect of the treatment of the sweet cherries with SAS-containing supernatants produced by *R. erythropolis* IMB Ac-5017 on their storage.

There are some publications related to applications of biological methods to treat post-harvested sweet cherry fruits, but only a few ones studied application of microbial SAS for the treatment of fruits (Dengle-Pulate et al., 2015; Jing and Bingbing, 2010). Thus, it was shown that post-harvested treatment of sweet cherry cultivar "Regina" with solution of rhamnolipids was more effective in comparison with washing with water (Golding, 2017). It was also shown that solutions of these SAS had antifungal activity *in vivo* on the causative agent of brown rot (*Monilinia fructicola*) and gray rot (*Botrytis cinerea*) in ripe fruits.

Application of solution of rhamnolipids synthesized by *Pseudomonas aeruginosa* LBI (1.0 g/L) for the treatment of Surinam cherry decreased the number of fungi cells on the fruit's surface by 6 times and bacterial cells by 33 times in comparison with washing with water (Dilarri et al., 2016). Concentrations of microbial SAS solutions used for the treatment of fruits and vegetables usually ranged from 1 to 3 g/L (Dilarri et al., 2016; Jing and Bingbing, 2010). Application of microbial SAS gave possibility to extend shelf-life of different fruits. The lemons treated with germicidal composition containing 2.5% sophorolipids synthesized by *Candida bombicola* ATCC 22214, sodium silicate as water softener, 1%, sodium carbonate as absorbing material, 1.5%, and polyethylene glycol as an antifoamer, 1%, did not show any signs of microbial spoilage after 7 days of storage (Dengle-Pulate et al., 2015). Spiking with the solution of sophorolipids, produced by strain *Wickerhamiella domercqiae* Y2A, with concentration of 3 g/L was proposed to prolong the preservation life of apples, pears, citrus fruits, and apricots at room temperature (Jing and Bingbing, 2010).

Combined usage of coating containing rhamnolipids (2% w/v) and chitosan (2% w/v) of sweet oranges extended the shelf life of fruits. Addition of chitosan increased the antimicrobial effect of rhamnolipids application against spoilage microorganisms on ripe oranges (Adetunji et al., 2015). The treatment with solution of lipopeptides (8 g/L), produced by *Bacillus methylotrophicus* XT1 CECT 8661, of grapes, strawberries and tomatoes infected with a common plant pathogen *Botrytis cinerea*, resulted in disease reductions by 100, 12 and 50%, respectively, after 6 days of incubation at 25 °C and 70% humidity (Toral et al., 2018). According to our results, SAS produced by *R. erythropolis* IMB Ac-5017 showed effective antimicrobial properties in concentrations 0.1 – 0.5 g/L (Figs. 1 and 2) that is lower than described in literature.

Microbial number on the surface of treated with supernatant sweet cherries depending on multipleness of the usage of SAS-containing supernatant of *R. erythropolis* IMB Ac- 5017

When sweet cherries are harvested, they pass a few successive stages before realization: hydrocooling shortly after harvest (if transporting in remote points will be necessary), sorting, washing, and packing (Quero-García et al., 2017). Solutions of mineral or organic substances to treat sweet cherries by immersion or sprinklings usually were used just one time (Chockchaisawasdee et al., 2016; Dilarri et al., 2016). For large scale treatment, sweet cherries more often are immersed in recirculated solution of sodium hypochlorite (Quero-García et al., 2017). Sodium hydrochloride has high antimicrobial activity and its usage is economically reliable, however its effectiveness is decreased during recirculation process due to the presence of organic impurities in water (Golding, 2017; Suslow, 2005). In our research, we studied the possibility of multiple usage of supernatant: the same SAS containing supernatant of *R.erythropolis* IMB Ac-5017 was used to treat three different groups of sweet cherries. The numbers of bacteria and fungi on the surface of fruits washed with water were $4.0 \cdot 10^3$ CFU/mL and $2.6 \cdot 10^3$ CFU/mL, respectively. It was shown that the amount of bacteria and fungi were higher when the same supernatant was used in the second and third time. After the third time of supernatant usage, the numbers of bacteria and fungi were almost the same as in the case when fruits were washed with water (Fig. 3). Application of supernatant with concentration of 0.5 g/L showed the best results: concentration of bacteria diminished after first usage of this supernatant by 10 times, after second usage it was diminished by 5 times and after third usage it was diminished by 3 times, meanwhile concentration of fungi diminished by 9, 5 and 4 times after I, II, and III usage of supernatant.

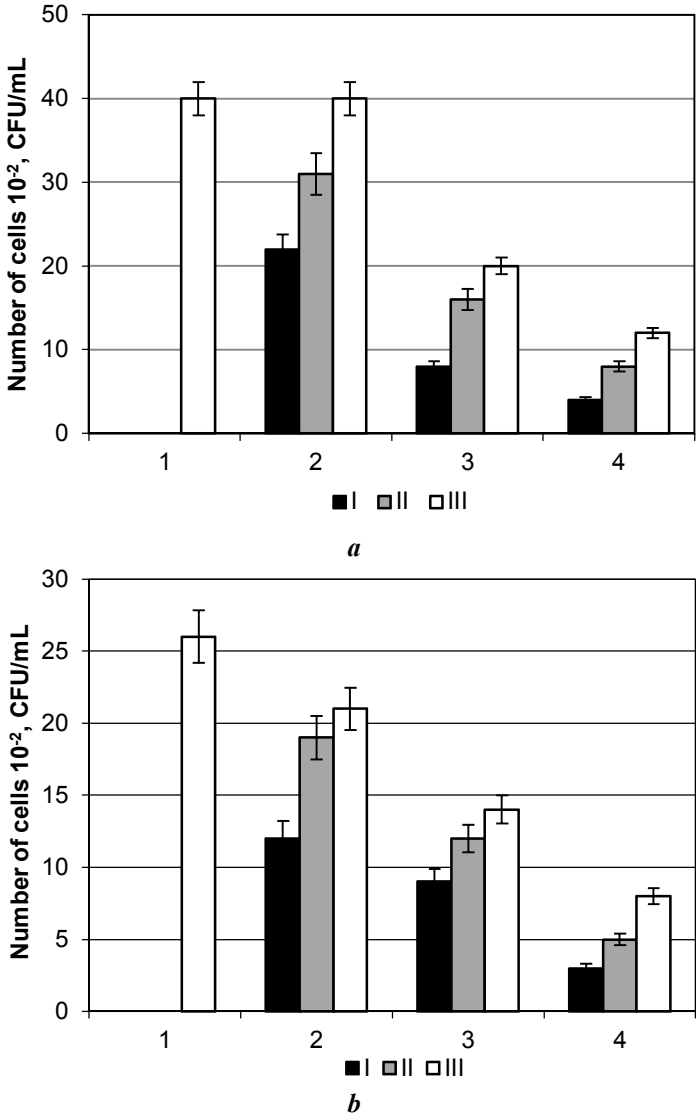


Figure 3. The total number of heterotrophic bacteria (A) and fungi (B) on the surface of sweet cherries washing with water (1); treated with supernatant of *R. erythropolis* IMB Ac-5017 with SAS concentration:
0.1 g/L (2); 0.2 g/L (3); 0.5 g/L (4) and different time of usage (I, II, III).

Antifungal activity of SAS-containing supernatant of *R. erythro*polis IMB Ac- 5017 on *Aspergillus niger,* infectious agents of postharvest spoilage of sweet cherry

One of the reasons for postharvest decay of sweet cherries is their contamination by fungi such as *Botrytis cinerea*, *Monilinia* spp., *Penicillium* spp., *Mucor* spp., *Rhizopus*

stolonifer, *Cladosporium* spp. and *Aspergillus niger*. Application of synthetic fungicides for postharvest treatment of sweet cherries allows managing post-harvest decay caused by these pathogens. However, their use is limited by fungicide regulatory issues in some countries (Project CY17000, 2017). For example, iprodione, which is active against brown rot of sweet cherries, is permitted to be used in Australia, but its usage is prohibited in the European Union countries because of its toxicity (Karabulut et al., 2001; Project CY17000, 2017). So, searching other substances with antimicrobial activity against certain plant pathogens is a subject of many studies (Sharma et al., 2018; Yan et al, 2016).

Antifungal activity of supernatant of *R. erythropolis* IMB Ac-5017 against *Aspergillus niger* is shown in Figure 4.

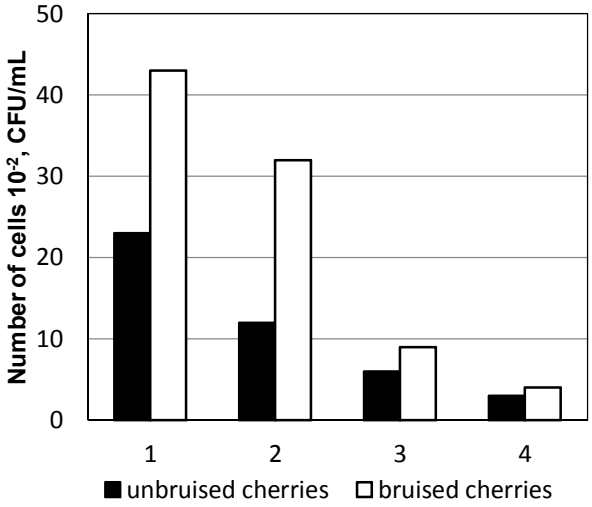


Figure 4. The number of cells *Aspergillus niger* P-3 on the surface of sweet cherry washing with water (1); treated with supernatant of *R. erythropolis* IMB Ac-5017 with SAS concentration: 0.1 g/L (2); 0.2 g/L (3); 0.5 g/L (4).

Sweet cherries in one group were bruised because cracks and fractures on the fruit's skins are possible entry sites for fungal infections. The number of fungi on the surface of untreated fruits was $8.6 \cdot 10^3$ CFU/mL. The number of cells of *A. niger* P-3 on the surface of unbruised and bruised sweet cherries washed with water diminished by 4 and 2 times, respectively. The number of cells of *A. niger* P-3 on the surface of unbruised and bruised sweet cherries treated with supernatant with SAS concentration 0.1%, 0.2 and 0.5% diminished in comparison with cherries washed with water by 2 and 1; 4 and 5; 7 and 11 times, respectively.

There are known a few researches concerning the application of microbial surface-active substances, most often solutions of rhamnolipids, in concentrations from 0.5 to 1.5 g/L, to treat artificially contaminated citrus fruits, potatoes and tomatoes (Sharma et al., 2016; Yan et al., 2014). Suppression of causative agents of potato rot *Fusarium solani* and tomatoes rot *Curvularia* sp. was observed after the treatment of vegetables with the solution containing 1 g/L of SAS produced by *Pseudomonas* sp. (Sharma et al., 2018). Effectiveness of application of solution of rhamnolipids (1.5 g/L) synthesized by *Pseudomonas aeruginosa*

JS29 to treat cherry tomatoes infected with *Alternaria alternata* was comparable with activity of synthetic fungicide carbendasim (Yan et al., 2016). There is information about application of rhamnolipids produced by P*seudomonas aeruginosa* to suppress growth of *Alternaria alternata* in lower concentration (0.5 g/L), but in combination with antagonistic of phytopathogens yeasts *Rhodotorula glutinis* (Yan et al., 2014). Treatment of tomatoes with solution of rhamnolipids (0.5 g/L) and suspension of yeasts *Rhodotorula glutinis* (1×10^8 cells/mL) decreased fungal contamination of tomatoes by *Alternaria alternata* by 60%.

According to our results, surface-active substances produced by *R. erythropolis* IMB Ac-5017 had high antifungal activity against *Aspergillus niger,* which is a causal agent of sweet cherries post-harvest spoilage, and could be used in concentrations lower than rhamnolipids described in literature.

Conclusion

Surface-active substances synthesized by *Rhodococcus erythropolis* IMB Ac-5017 have high antimicrobial activity in concentration lower then known microbial SAS and could be used as supernatant without extraction and purification for treatment of sweet cherry to extend their shelf-life. SAS – containing supernatant was effective even in the case of its reuse.

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