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SYNERGISTIC EFFECT OF SURFACTANTS OF *NOCARDIA VACCINII* IMV B-7405 AND ESSENTIAL OILS ON *CANDIDA* GENUS YEAST

The increase in the number of resistant strains of Candida genus representatives, capable of forming biofilms on various surfaces, stimulates the search for new, alternative methods of combating them, one of which is the use of compounds of natural origin, such as essential oils. However, at the same time, their concentration should be minimal, which is due to the ability of essential oils to cause severe damage of the human's central nervous system and aspiration pneumonia. This leads to the necessity of searching for new methods to reduce the concentration of essential oils and at the same time to preserve their properties, in particular, by their use in a mixture with other antimicrobial agents, which can be microbial surfactants. Previously, it was found that the degree of yeast biofilm destruction under the action of Nocardia vaccinii IMV B-7405 surfactants dependson the nature of the growth substrate and is the highest in the presence of preparations synthesized on purified glycerol. ***Aim.*** *To study the synergism of antifungal activity and the role in the destruction of biofilms of a mixture of Nocardia vaccinii IMV B-7405 surfactants synthesized on glycerol of different quality and essential oils.* ***Methods.*** *N. vaccinii IMV B-7405 was grown in a medium containing purified glycerol or waste from biodiesel production at a concentration of 2% (v/v) as carbon sources. The surfactants were extracted from the supernatant of cultural liquid by a modified Folch mixture. The antimicrobial activity of essential oils, surfactants, and their mixtures was determined by the index of the minimum inhibitory concentration. To assess the synergistic effect of a mixture of surfactants with essential oils, the fractional inhibitory concentration index was used. The degree of biofilm destruction (%) was determined as the difference between the cell adhesion in untreated and treated with surfactants, essential oil, or their mixture wells of the polystyrene microplates.* ***Results****. It was found that the surfactants synthesized by N. vaccinii IMV B-7405 on both purified glycerol and waste from biodiesel production showed synergistic antifungal activity in mixtures with cinnamon and lemongrass essential oils. Thus, the minimum inhibitory concentrations against Candida*

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albicans D-6, Candida utilis BVS-65, and Candida tropicalis RE-2 of a mixture of surfactants synthesized on purified glycerol with cinnamon and lemongrass essential oils were 1.8—7.5 and 3.7— 15 μg/mL, respectively, and were lower than in the case of using surfactants (30-60 μg/mL), cinnamon or lemongrass essential oil (156—312 μg/mL) alone. The use of a mixture of surfactants obtained on waste from biodiesel production and cinnamon or lemongrass essential oils made it possible to reduce the minimum inhibitory concentrations of the latter against studied yeast test cultures by 14—56 times. At the same time, the index of fractional inhibitory concentration did not exceed 0.5, which indicates the synergism of the antifungal activity of the mixture of these compounds. The destruction of Candida yeast biofilms under the action of surfactants synthesized on both purified glycerol and waste from biodiesel production in a mixture with cinnamon or lemongrass essential oils reached 60—67 and 67—77%, respectively, which is an average of 25—35% higher compared to the use of each monopreparation separately. ***Conclusions.*** *The results presented in this paper confirm the previously obtained data that N. vaccinii IMV B-7405 surfactants, synthesized on both traditional substrates and toxic industrial wastes, have antimicrobial and antiadhesive synergistic action with essential oils, which allows us to consider them as potential components of the so-called «antifungal locks» in the fight against of Candida genus representatives.*

Keywords: *Nocardia vaccinii IMV B-7405, surfactants, essential oils, yeast of Candida genus, synergism of antifungal activity, destruction of biofilms.*

In recent years, the number of reports has increased about the spread of resistant *Candida* strains [1, 2], 93% of which are resistant to fluconazole, 35% and 7% to amphotericin B and echinocandins respectively. This is mainly due to the use of only four classes of antifungal drugs in the hospital practice, namely azoles (fluconazole, itraconazole, isavuconazole, posaconazole, and voriconazole), polyenes (amphotericin B and its lipid derivatives), echinocandins (anidulafungin, caspofungin, and pyrimidine analog lucitosine [3], and the ability of the *Candida* genus representatives (mainly *C. alibicans*) to form biofilms on the surface of mucous membranes and implanted medical devices [4, 5]. Such a situation creates a necessity in new, alternative to antibiotics, antimicrobial drugs, which can be essential oils (EO). It is known that the presence of alcohols, aldehydes, and phenols in their composition makes them promising antimicrobial, antifungal, and antiviral agents that can be used in many industries [6, 7]. However, at the same time, their concentration should be minimal due to the ability of EO to cause severe damage of human's central nervous system and aspiration pneumonia [8]. This leads to the necessity of searching for new methods to reduce the EO concentration, and at the same time to preserve their properties, in particular, via their use in a mixture with other antimicrobial agents, which can be microbial surfactants.

Previously [9, 10], the synergism of antimicrobial activity and role in biofilms destruction of a mixture of surfactants synthesized by *Nocardia vaccinii* IMV B-7405 on oil-containing industrial waste with EO against a wide range of microorganisms has been found. However, those findings and the results of our research presented in [11] showed that in the presence of surfactants synthesized on waste oil, the degree of yeast biofilms destruction did not exceed 25—40%, while under the action of surfactants obtained on purified glycerol, the destruction of such biofilms was significantly higher and reached 52—72%.

In connection with the above, the aim of this work is to study the synergism of antifungal activity and the role in the destruction of biofilms of a mixture of *Nocardia vaccinii* IMV B-7405 surfactants synthesized on glycerol of different quality and EO.

Materials and methods. The object of the research is *Nocardia vaccinii* K-8 strain, registered in Depositary of Microorganisms of the Zabolotnyi Institute of Microbiology and Virology of NAS of Ukraine under the number IMV B-7405.

The fungal strains *Candida albicans* D-6, *Candida tropicalis* RE-2, and *Candida utilis*

BVS-65 (isolated from oral mucosa and skin) from the collection of cultures of the Department of Biotechnology and Microbiology of the National University of Food Technologies were used as test cultures in determining the antimicrobial activity of surfactants, EO, and their mixture as well as their role in the destruction of biofilms. EO lemongrass (Aromatica LLC, Ukraine) and cinnamon (RosCosmetics LLC, Ukraine) were used.

N. vaccinii IMV B-7405 was grown in a liquid mineral medium of the following composition (g/L): $NaNO_3$ — 0.5; $MgSO_4 \cdot 7H_2O$ — 0.1; $CaCl \cdot 2H_2O$ — 0.1; KH_2PO_4 — 0.1; $FeSO_4 \cdot 7H_2O$ — 1, and yeast autolysate — 0.5 v/v. Purified glycerol and waste from biodiesel production (biofuel plant, Poltava oblast) at a concentration of 2 v/v were used as substrates.

The quantity of extracellular surfactants was determined using the modified-by-us method of Bligh and Dyer after extraction with a mixture of chloroform and methanol (2:1) from the supernatant of the culture liquid as described in the study [9, 10].

The antimicrobial action of EO, surfactants, and their mixtures was determined by the index of the minimum inhibitory concentration (MIC) [12] as described in the article [9]. The results were evaluated visually by the medium turbidity: (+) — for tubes in which the medium turbidity was observed (growth of the testing culture), (–) — no turbidity (no growth). The MIC for the surfactant solution or essential oil was defined as the concentration of surfactants in the last tube where growth was absent.

The synergistic effect of surfactants and EO was evaluated by the indicator of fractional inhibitory concentration (FIC) as the sum of the ratios of the concentration of each substance in a mixture to its MIC, using the formula [13]:

$$\Sigma = FIC = (C_A/MIC_A) + (C_B/MIC_B),$$

where $C_{A, B}$ is the concentration of the antimicrobial substance in the mixture;

$MIC_{A, B}$ is the minimum inhibitory concentration of the antimicrobial substance (A or B) used separately.

The ratio of drugs in the mixture was 1:1, the concentration of surfactants remained unchanged, and the oil concentration was reduced by the method of two-fold serial dilutions; in another embodiment, the concentration of essential oil remained unchanged, and the concentration of surfactants was reduced.

To obtain biofilm, 180 μL of liquid wort and 20 μL of one-day test culture suspension were added into polystyrene microplates and incubated for 24 hr at the optimal temperature. Then the cultural liquid was poured off, and again 180 μL of fresh liquid wort and 20 μL of the suspension of the test culture were added and incubated for 24 hr. After 48 hr, the cultural liquid was poured off, and into each of the wells of the microplates (pre-covered with a biofilm), 200 μL of preparations (surfactants and EO in the ratio 1:1) with different concentrations were added. For the control wells, surfactant preparations were replaced with distilled tap water (200 μL). After 24 h of exposition, the wells were thrice washed with 200 μL of distilled water, and the number of adherent cells was determined spectrophotometrically. The degree of biofilm destruction (%) was determined as the difference in cell adhesion between untreated and treated (with surfactants, EO, or their mixture) wells in the polystyrene microplates.

All experiments were performed in three replications; the number of parallel determinations in experiments ranged from three to five. Statistical processing of the experimental data was performed as described in the previous paper [12]. The average difference was considered significant at $P < 0.05$.

Results. It was found that, regardless of the nature of the substrate used, *N. vaccinii* IMV B-7405 surfactants showed synergistic antimicrobial activity with both studied EO (Tables 1 and 2). For example, the MICs for surfactants

synthesized on waste from biodiesel production against *C. tropicalis* RE-2-2 and *C. utilis* BVS-65 were 22.1 and 44.2 μg/mL, the MIC for cinnamon EO was 156 μg/mL, while for their mixtures — 5.5 and 11.0 μg/mL, respectively.

The use of surfactants formed on purified glycerol in combination with cinnamon EO made it possible to reduce the MIC of the latter against the studied yeast test cultures from 156 and 312 μg/mL down to 1.8 and 3.75 μg/mL, respectively (Table 1).

Similar patterns were observed when using a mixture of IMV B-7405 strain surfactants and lemongrass EO. So, regardless of the carbon source in the *N. vaccinii* IMV B-7405 cultivation medium, the synthesized surfactants in combination with lemongrass essential oil reduced the MIC of the latter against *C. albicans* D-6, *C. utilis* BVS-65, and *C. tropicalis* RE-2-2 from 165—312 μg/mL to 2.7—15 μg/mL (Table 2). At the same time, the index of FIC (with the exception of the data on *C. albicans* D-6), did not exceed 0.5, which indicates synergism.

Table 1. **Antifungal activity of *N. vaccinii* IMV B-7405 surfactants, cinnamon essential oil, and their mixtures**

Substrate for surfactant synthesis	Test culture	MIC (μg/mL)				FIC, FIC ≤ 0.5 synergy
		Surfactants	Cinnamon EO	Surfactants in a mixture with cinnamon EO*	Cinnamon EO in a mixture with surfactants **	
Purified glycerol	*Candida albicans* D-6	30	312	19.5	3.75	0.2
	Candida tropicalis RE-2	30	312	9.7	1.8	0.1
	Candida utilis BVS- 65	60	156	39	7.5	0.37
Waste from biodiesel production	*Candida albicans* D-6	22.1	156	39	5.5	0.5
	Candida tropicalis RE-2	22.1	312	19.5	5.5	0.37
	Candida utilis BVS-65	44.4	156	19.5	11	0.37

Table 2. **Synergism of the antifungal action of *N. vaccinii* IMV B-7405 surfactants and lemongrass essential oil**

Substrate for surfactant synthesis	Test culture	MIC (μg/mL)				FIC, FIC ≤ 0.5 synergy
		Surfactants	Lemongrass EO	Surfactants in a mixture with lemongrass EO*	Lemongrass EO in a mixture with surfactants **	
Purified glycerol	*Candida albicans* D-6	30	156	39	15	0.75
	Candida tropicalis RE-2	30	312	2.4	3.75	0.13
	Candida utilis BVS- 65	60	156	1.2	3.75	0.07
Waste from biodiesel production	*Candida albicans* D-6	22.1	156	1.2	5.5	0.25
	Candida tropicalis RE-2	22.1	312	1.2	2.7	0.12
	Candida utilis BVS-65	44.4	156	39	5.5	0.37

Note: The error of the analysis of MIC did not exceed 5%; Tables 1 and 2: * — the concentration of essential oil remained unchanged, and the concentration of surfactants reduced by the method of two-fold serial dilutions; ** — the concentration of surfactants remained unchanged, and the concentration of the oil reduced by the method of two-fold serial dilutions.

At the next stage, the possibility of using surfactant strain IMV B-7405 in a mixture with EO for the destruction of biofilms was investigated.

Table 3 shows that surfactants synthesized by *N. vaccinii* IMV B-7405 on both purified glycerol and waste from biodiesel production exhibit synergism in yeast biofilms destruction in a mixture with cinnamon and lemongrass EO in a wide range of concentrations 6—300 μg/mL, however, the highest degree of biofilms destruction was achieved at a concentration of 37.5 μg/mL. Thus, with using a mixture of surfactant and EO (37.5 μg/mL) solutions, the degree of *C. albicans* D-6, *C. utilis* BVS-65, and *C. tropicalis* RE-2-2 biofilm destruction is within 60.4—76.5%, that is, is higher than with using only surfactants (42.3—49.9%), cinnamon oil (37.4—40.7%) or lemongrass (31.7—36.2%) in similar concentrations.

Discussion. By March 2022, there had been about 1,500 papers in the PubMed [https://pubmed.ncbi.nlm.nih.gov/] information search database for the keywords «antifungal activity and EO», of which more than a third were published from 2010 to 2021 and relate to the use of EO/plant extracts against clinical isolates of *Candida* genus. The increase in the number of such works is due to several factors. Firstly, in people with a weakened immune system, especially against the background of the development of the human immunodeficiency virus (HIV), cancer chemotherapy, and the use of broad-spectrum antibiotics, representatives of *Candida* genus can cause severe infectious diseases and form biofilms [14—16]. Secondly, clinically important antifungal agents, such as fluconazole or amphotericin B, flucytosine, echinocandins, itraconazole or ketoconazole, are almost ineffective against pathogens (especially *C. albicans*), while their concentrations required to reduce the metabolic activity of cells in biofilms are 5—8 times higher than the corresponding MIC against planktonic forms [17]. Thirdly, due to the multi-component composition (including the presence of phenolic terpenoids), EOs are effective antifungal agents, including in the fight against biofilms [15, 16]. However, a significant disadvantage of EOs as antimicrobial agents is their high MIC (up to several mg/mL), which can be reduced by using oils in a mixture with surfactants of microbial origin.

This work is a logical continuation of our previous studies of the biological activity of surfactants synthesized by *N. vaccinii* IMV B-7405 on toxic industrial wastes and the synergism of the antimicrobial and antiadhesive action of a mixture

Table 3. **Destruction of yeast biofilms under the action of *N. vaccinii* IMV B-7405 surfactants, essential oils, and their mixtures**

Substrate for surfactant synthesis	Test culture	Destruction (%) of biofilm under the action of				
		Surfactants	Lemongrass EO	Cinnamon EO	Mixture of surfactants and lemongrass EO	Mixture of surfactants and cinnamon EO
Purified glycerol	*Candida albicans* D-6	42.4	31.7	37.4	63.2	69.3
	Candida tropicalis RE-2	45.2	36.2	40.2	64.1	71.5
	Candida utilis BVS-65	43.9	33.9	40.7	64.3	69.3
Waste from biodiesel production	*Candida albicans* D-6	47.8	31.7	37.4	65.6	67.4
	Candida tropicalis RE-2	42.3	36.2	40.2	60.4	68.7
	Candida utilis BVS-65	49.9	33.9	40.7	66.9	76.5

Note: The concentration of solutions of surfactants and EO in mono- and mixed preparations was 37.5 μg/mL. When determining the degree of biofilm destruction, the error did not exceed 5%.

of surfactants with various biocides [9—11, 18]. The choice of waste oils and technical glycerol as substrates allows one not only to reduce the cost of surfactants through the use of cheap and available large quantities of these industrial wastes but also to preserve the environment through the disposal of hazardous toxic waste. In addition, to date, there have been no data in the literature on the synergistic antimicrobial effect of surfactants synthesized on waste oil or waste from biodiesel production with other antimicrobial compounds. Note that since the publication of the works [9, 10], we have been unable to find in the available literature information on the synergism of biofilm destruction under the action of a mixture of microbial surfactants and EO, as well as on the synergism of their action on *Candida* yeast.

In a previous work [9], we noted that the specific effect of EO on bacteria or yeast depends on their component composition. Thus, cinnamaldehyde and limolene, which are the main components of cinnamon and lemongrass oils respectively, inhibit electron transport and change protein translocation and synthesis of cellular components in yeast. Comparison of the synergistic antifungal action of cinnamon and lemongrass oils mixed with the *N. vaccinii* IMV B-7405 surfactant synthesized from various industrial wastes showed that surfactants obtained on waste from biodiesel production were more effective in reducing MIC than ones obtained on waste oil: MIC against the studied yeast test cultures decreased by 14—56 (see Tables 1 and 2) and 8—15 times [9], respectively.

Note that there is no information in the literature on the synergism of the antimicrobial activity of microbial surfactants and EO of cinnamon and lemongrass. At the same time, there are works concerning the possibility of using a mixture of two or more EOs [19], a mixture of EO with antifungal drugs (mainly fluconazole) [20—24], including cinnamon [21, 22] and lemongrass [23, 24]. The studies, the results of which are presented in [20—24], are quite relevant, especially considering the spread of fluconazole-resistant strains among representatives of *Candida* genus. Thus, in the presence of cinnamon EO (MIC 62.5 μg/mL) in a mixture with fluconazole (MIC 1000 μg/mL), a decrease in the order of the MIC of the antibiotic against *C. albicans* ATCC 10213 was observed, and the FIC value was 0.5 indicating their synergy [21]. The use of a mixture of EOs of cinnamon (200 μg/mL) and fluconazole (25 μg/mL) reduced the concentrations of EO and antibiotic by 8 and 4 times, respectively [22]. In [23], it was found that the components of lemongrass EO (limolen, nerol, citral, linalool, geraniol, citraniol, and 1,8-cineole) show a synergism of antifungal activity in a mixture with fluconazole against *C. albicans* ATCC90028. For example, the MIC of fluconazole and limolen was 0.1 and 2 mg/mL, while in the mixture — 0.1 and 0.5 mg/mL, respectively. The FIC value was 0.4, which indicates synergism between the compounds' activities [23]. Similar patterns were observed in the case of a combination of citral (0.25 mg/L) and fluconazole (256 mg/L) against *C. albicans* UPV 15-157, with an FIC value of 0.5 [24].

Given the presence of phenolic terpenoids in the composition of cinnamon and lemongrass EOs, in addition to their antifungal activity against planktonic forms, they are able to destroy biofilms. Thus, one of the established mechanisms of their action is a decrease in the thickness of the cell wall in representatives of *Candida* genus due to inhibition of β-1-3-glucansynthase [17].

At the same time, the researchers note that EOs are able to destroy yeast biofilms only at certain stages of their formation. Thus, citral, one of the main components of lemongrass EO, is quite effective for destroying yeast biofilms at the early stages of formation (up to 24 hr) [24]. The destruction of *C. albicans* ATCC90028 biofilm formed within 48 hr is possible only with a mixture of citral and fluconazole (effective concentrations of citral and antifungal agent 0.139 and 0.08 mg/mL, respectively) [23].

Our studies have shown the possibility of using lemongrass EO in combination with *N. vaccinii* IMV B-7405 surfactants for the destruction of *Candida* biofilms formed within 48 h (see Table 3). The effective concentration of components in the mixture, which provided a high degree of biofilm destruction (60—67%), was only 37.5 μg/mL, that is, significantly lower than described in [23]. It should be noted that the percentage of biofilms destruction under the action of monopreparations of lemongrass EO and surfactants obtained on both purified glycerol and waste from biodiesel production in the same concentration did not exceed 32—36 and 42—50%, respectively.

A somewhat higher degree of biofilm destruction (67—77%) was observed when using a mixture of *N. vaccinii* IMV B-7405 surfactants synthesized on glycerol of different quality and cinnamon EOs (Table 3). In our opinion, this may be due to the presence of cinnamaldehyde in the composition of cinnamon oil, which is an effective agent in the biofilm destruction [6].

Conclusions. The results presented in this paper confirm the previously obtained data that *N. vaccinii* IMV B-7405 surfactants, synthesized on both traditional substrates and toxic industrial wastes, have an antimicrobial synergistic effect when combined with EO, which allows us to consider them as potential components of the so-called «antifungal locks» in the fight against representatives of the *Candida* genus.

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СИНЕРГІЧНА ДІЯ ПОВЕРХНЕВО-АКТИВНИХ РЕЧОВИН *NOCARDIA VACCINII* ІМВ В-7405 ТА ЕФІРНИХ ОЛІЙ НА ДРІЖДЖІ РОДУ *CANDIDA*

Збільшення кількості резистентних штамів представників роду *Candida*, здатних до утворення біоплівок на різноманітних поверхнях стимулює пошук нових, альтернативних методів боротьби з ними, одним з яких є використання сполук природного походження, зокрема ефірних олій. Але при цьому концентрація олій повинна бути мінімальною, що пов'язано з їхньою здатністю спричиняти серйозні ураження центральної нервової системи людини та аспіраційну пневмонію. Це, у свою чергу, зумовлює необхідність пошуку шляхів зниження концентрації ефірних олій без втрати їхньої антимікробної активності, що досягається використанням цих сполук у суміші з іншими біоцидами, наприклад, мікробними поверхнево-активними речовинами. Раніше було встановлено, що ступінь деструкції дріжджових біоплівок за дії поверхнево-активних речовин (ПАР) *Nocardia vaccinii* IMV B-7405 залежить від природи ростового субстрату і є найвищим за наявності препаратів, синтезованих на очищеному гліцерині. **Мета.** Дослідити синергізм антифунгальної активності та ролі у руйнуванні біоплівок суміші ПАР *N. vaccinii* IMV B-7405, синтезованих на гліцерині різної якості, та ефірних олій. **Методи**. *N. vaccinii* ІМВ В-7405 вирощували у середовищі, що містило як джерело

вуглецю очищений гліцерин та відходи виробництва біодизелю у концентрації 2 об.%. ПАР екстрагували із супернатанту культуральної рідини модифікованою сумішшю Фолча. Антимікробну активність ефірних олій, ПАР та їх сумішей визначали за показником мінімальної інгібуючої концентрації. Для оцінки синергічного ефекту суміші ПАР з ефірними оліями розраховували індекс фракційної інгібуючої концентрації. Ступінь руйнування біоплівки (%) визначали як різницю між адгезією клітин в необроблених і оброблених ПАР, ефірною олією або їх сумішшю в лунках полістиролового мікропланшета. **Результати.** Встановлено, що ПАР, синтезовані *N. vaccinii* IMB B-7405, проявляли синергізм антифунгальної активності у суміші з ефірними оліями кориці і лемонграсу як на очищеному гліцерині, так і відходах виробництва біодизелю. Так, мінімальні інгібуючі концентрації щодо *Candida albicans* Д-6, *Candida utilis* БВС-65 та *Candida tropicalis* РЕ-2 суміші ПАР , синтезованих на очищеному гліцерині, з ефірною олією кориці та лемонграсу становили відповідно 1.8—7.5 і 3.7—15 мкг/мл та були нижчими, ніж за дії тільки ПАР (30—60 мкг/мл), ефірної олії кориці чи лемонграсу (156—312 мкг/мл). Використання суміші ПАР, одержаних на відходах виробництва біодизелю, та ефірних олій кориці і лемонграсу дало змогу знизити мінімальні інгібуючі концентрації останніх щодо досліджуваних дріжджових тест-культур у 14—56 разів. При цьому показник фракційної інгібуючої концентрації не перевищував значення 0,5, що вказує на синергізм антифунгальної активності суміші цих сполук. Деструкція біоплівок дріжджів роду *Candida* за дії ПАР, синтезованих як на очищеному гліцерині, так і відходах виробництва біодизелю, у суміші з ефірною олією кориці та лемонграсу досягала відповідно 60—67 і 67—77%, що в середньому на 25—35% вище порівняно із застосуванням кожного з монопрепаратів окремо. **Висновки.** Наведені у даній роботі результати підтверджують отримані раніше дані про те, що ПАР *N. vaccinii* IMV B-7405, синтезовані як на традиційних субстратах, так і токсичних промислових відходах, проявляють синергічну з ефірними оліями антимікробну дію, що дає змогу розглядати їх як потенційних складових так званих «антифунгальних замків» у боротьбі з представниками роду *Candida.*

Ключові слова: *Nocardia vaccinii IMB B-7405, поверхнево-активні речовини, ефірні олії, дріжджі роду Candida, синергізм антифунгальної активності, руйнування біоплівок.*