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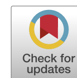
Optimization of laboratory cultivation conditions for the synthesis of antifungal metabolites by bacillus subtilis strains



Tatyana M. Sidorova, Anzhela M. Asaturova, Anna I. Homyak *, Natalya A. Zhevnova, Margarita V. Shternshis, Natalia S. Tomashevich

Federal State Budgetary Scientific Institution (FSBSI), "All-Russian Research Institute of Biological Plant Protection", 350039, Krasnodar-39, VNIIBZR, Russia

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ABSTRACT

In order to achieve the optimal number of colony forming units and a high level of antifungal metabolites synthesis, we carried out the periodic cultivation of the *Bacillus subtilis* BZR 336 g and *Bacillus subtilis* BZR 517 strains at various pH and temperature levels. In the experiment for determining the optimal temperature, the maximum titer of *B. subtilis* BZR 336 g bacterium (1.6–1.7 × 10^9 CFU/ml) was recorded at a cultivation temperature of 20–25 °C. For *B. subtilis* BZR 517 strain, the temperature turned out to be optimal at 30 °C: the titer was 8.9 × 10^8 CFU/ml. The maximum antifungal activity of *B. subtilis* BZR 336 g strain against the test culture of *Fusarium oxysporum* var. *orthoceras* was observed at a cultivation temperature of 20–25 °C; for *B. subtilis* BZR 517 strain, 25–30 °C. When determining the optimal pH level, it was found that a high titer of *B. subtilis* BZR 336 g strain cells was determined at pH 8.0 (2.7 × 10^9 CFU/ml), for *B. subtilis* BZR 517 strain it was at pH 6.0–8.0 (1.0 × 10^9 CFU/ml). The maximum antifungal activity was noted with the same indicators. Chromatographic and bioautographic analyses suggest that the synthesized antifungal metabolites belong to surfactin and iturin A. The data obtained in this research can be used in the development of the technology for the production of effective biofungicides to protect crops against *Fusarium* pathogens.
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1. Introduction

One of the current trends in the biological plant protection is the development and application of biological products based on antagonistic bacteria that can synthesize a wide range of antibiotics and other metabolites active against bacteria and fungi that cause plant diseases in agriculture (Lirong et al., 2015; Vejan, et al., 2016; Backer et al., 2018; Nawaz et al., 2018).

Antimicrobial activity of bacteria of the genus *Bacillus* largely depends on their ability to produce metabolites, which are mostly represented by antibiotics of the polypeptide and aminoglycoside series (Farace et al., 2015; Toral et al., 2018), suppressing both the growth and development of a large number of harmful organisms (Wang et al., 2015; Torres et al., 2017). Depending on the strain, bacteria of the genus *Bacillus* can be producers from 50 to 200 different biologically active substances. It is a wide range of such compounds that effectively reduces the number of pathogens (Debois et al., 2015; Mnif et al., 2016; Harwood et al., 2018; Li et al., 2019).

However, it should be mentioned that only some fungicidal factors of bacterial origin are isolated in pure form and characterized. In some cases, the mechanism of the action of microorganisms on phytopathogenic fungi has not been studied. The lack of data on physiological and biochemical properties and the mechanism of the inhibitory effect of fungicidal bioagents makes it difficult to develop highly effective biological products for protecting plants against pathogens (Kumar and Singh, 2015; Glare et al., 2016). Some of the best known biologically active plant protection compounds are cyclic lipopeptides, which include the surfactin, iturin, and fengicin families and have different activity levels. In addition to direct antagonistic activity, the family of surfactins also induces the resistance of many hosts to various diseases, stimulating the

* Corresponding author at: FSBSI "All-Russian Research Institute of Biological Plant Protection", 350039, Krasnodar-39, VNIIBZR, Russia.

E-mail addresses: 0166505@mail.ru (T.M. Sidorova), biocontrol-vniibzr@yandex.ru (A.M. Asaturova), HomyakAI87@mail.ru (A.I. Homyak), tiamat-7@mail.ru (N.A. Zhevnova), shternshis@mail.ru (M.V. Shternshis), tom-s2@yandex.ru (N.S. Tomashevich).

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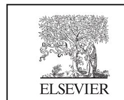

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immune responses of plants. For example, purified surfactin triggers defense reactions in cell suspensions of grapes and tobacco and causes systemic resistance to *B. cinerea* in tomato plants. The fengicin family mainly exhibits antifungal activity. Unlike surfactin, the protection caused by fengicin is specific to certain plant species or host-pathogen systems. For example, fengicin produced by *B. subtilis* BBG111 plays an important role in the induced protective state of rice (*Oryza sativa* L.) against Rhizoctonia solani (Bartolini et al., 2019). The iturin family has strong antifungal activity, but limited antiviral and antibacterial activity, which indicates the ability of these lipopeptides to be responsible for its biocontrol activity against fungal infections. Variants and ratios of lipopeptides synthesized by different species and strains of *Bacillus* may vary.

In addition, it should be taken into consideration that at the stage of obtaining a biological product, the degree of biologically active metabolites accumulation is directly dependent not only on the individual characteristics of the producer microbial strain, but also on the conditions for obtaining the target product (Abada et al., 2014; Yi et al., 2015). Among the most significant factors affecting the manifestation of the antibiotic properties of microorganisms are the composition of the medium, acidity, temperature, time of cultivation, etc. (Volova et al., 2014; Tumbarski et al., 2015).

So the studies showed that some carbon sources are focused on the synthesis of only one lipopeptide family, while most nitrogen sources allow high joint production of fengicin and surfactin. Moreover, temperature, pH, and oxygenation influenced their biosynthesis depending on nutritional conditions (Yaseen et al., 2017). These data are proved by the studies where it was found that a high concentration of glucose (50–60 g/l) can lead to the accumulation of excess glucose in the medium, which leads to a low pH and, as a result, a decrease in B. subtilis production of surfactin (Chen et al., 2015). In addition, changes in pH can significantly affect the resistance of B. subtilis spores to temperature changes and their hydrophobic characteristics (Eschlbeck et al., 2017).

It should also be taken into account that, compared with synthetic chemical fungicides, the commercialization of biofungicides is limited by lower yields of active metabolites and higher production costs. It is estimated that substrates account for 10–30% of the total production costs related to fermentation processes. Thus, the determination of optimal conditions and the search for inexpensive raw material substrates will reduce production costs (Li et al., 2016).

However, despite extensive efforts to study the effect of various cultivation conditions on the total production of antifungal lipopeptides, there is only limited knowledge about the effect of these parameters on the production of various variants and their relative amounts in the lipopeptide mixture. The lack of data on the physiological and biochemical properties and the mechanism of the inhibitory effect of fungicidal bioagents makes it difficult to create highly effective biological preparations for protecting plants against pathogens (Hashem et al., 2019).

Thus, one of the current research areas today is the search for optimal conditions for the cultivation of biological products producer strains: improving cultivation methods, studying the viability and activity of microorganisms under various cultivation conditions, taking into account their trophic needs and physiological and biochemical characteristics. Moreover, the development of the preparations requires understanding of the biological role of microbial metabolites as inducers of plant resistance to stress factors. Improving the methods for the isolation and purification of secondary metabolic compounds is important for the subsequent decoding of their chemical nature.

The aim of our research was to study the optimal parameters of the cultivation of *B. subtilis* BZR 336 g and *B. subtilis* BZR 517 strains, promoting active bacterial growth, increasing their antagonistic activity and accumulation of antifungal compounds and the maximum evidence of the ability of the studied strains to produce antifungal metabolites.

The aim of the research was to study the temperature and acidity of the medium to optimize the cultivation conditions of *B. subtilis* BZR 336 g and *B. subtilis* BZR 517 strains according to two criteria: the number of colony forming units and antifungal activity against *F. oxysporum* var. *orthoceras* BZR 6 under the conditions of periodic cultivation.

2. Materials and methods

2.1. Bacterial strains and test-culture

Bacillus subtilis BZR 336g and *Bacillus subtilis* BZR 517, isolated from winter wheat rhizoshere were used in the experiments. These bacterial strains belong to the Bioresource Collection of the «State Collection of Entomoacariphages and Microorganisms» of All-Russian Research Institute of Biological Plant Protection, Krasnodar, Russia (http://ckp-rf.ru/ № 585858). The strains were stored on nutrient agar at 5 °C and were subcultured every three months. Phytopathogenic fungus (monosporous isolate) *F. oxysporum* var. *orthoceras* App. et Wr. BZR 6 was used as a test-culture.

In our research we used Unique Scientific Facility «New generation technological line for developing microbiological plant protection products» of All-Russian Research Institute of Biological Plant Protection, Krasnodar, Russia (http://ckp-rf.ru/ № 671367).

2.2. Optimal conditions for the cultivation of laboratory samples of strains

To determine the optimal cultivation temperature, the strains were incubated at 20.0, 25.0, 30.0 and 35.0 °C. To choose the optimal acidity of the medium, the strains were grown on a liquid CM, g/l KCl – 0.5, $MgSO_4{\cdot}7H_2O$ – 0.5, $K_2HPO_4{\cdot}3H_2O$ – 1.0, $CaCO_3$ – 3.0, $FeSO_4{\cdot}7H_2O$ – 0.01, corn extract– 2.0, molasses – 20.0, at optimum temperatures. The main components of the original nutrient medium were selected as a result of the previous research by the authors (Asaturova et al., 2015). By adding lactic acid or alkali (4 N NaOH solution), the medium pH was adjusted to 3.0, 6.0, 8.0, and 10.0 using a Sartorius PB-11 pH -meter (Germany). All experiments were replicated three times. For all experiments, a liquid culture was obtained by the method of periodic cultivation. Incubation was carried out in thermostated cell cultivation systems (180 rpm) "New Brunswick Scientific Excella E25" (USA) for 48 h. Periodic cultivation was carried out in conical flasks (350 ml) with a nutrient medium volume of 100 ml and preliminary introduction of stock culture (2% of the nutrient medium volume). Stock culture was obtained by introducing agar blocks with the studied strains into conical flasks and subsequent cultivation.

At the end of cultivation, the number of bacterial cells was determined by the Koch method in all experiments (Netrusov, 2005). The grown colonies were counted with the Color Qcount, Spiral Biotech, 530 (USA) system for the automatic counting colonies.

Antibiotic activity of the strains was determined by the dilution method (Egorov, 2004). The relationship between the fungus and the bacteria such as the presence or absence of zones, their size, change in the color of the pathogen mycelium was noted. The degree of inhibition of pathogen mycelium growth was determined by the formula (Montealegre et al., 2003):

$$I = (1 - (A/B)) \times 100, \quad (1)$$

where

I – inhibition of pathogen mycelium growth, %;
A – fungal growth under *B. subtilis* action, mm;
B – fungal growth in control, mm.

2.3. Chromatography and bioautography

After the cultivation, the liquid culture of *B. subtilis* was centrifuged at 10^4 rpm within 20 min at Centrifuge 5810R, Eppendorf (Germany), the supernatant was extracted with three volumes of ethyl acetate for 1 h. The obtained extract was evaporated, the dry residue was washed with a minimum amount of ethyl acetate and used for the further study. As standards for antifungal lipopeptides, commercial surfactin and iturin A reagents (Sigma-Aldrich company) are used. Isolation and analysis of the synthesized metabolites and commercial lipopeptides was performed using the method of ascending thin layer chromatography, which was carried out on silica gel plates (Merck company (Germany), 2 mm thick layer. The solvent phase was the ethyl acetate-ethanol–water solvent system (40:15:15). After analyzing the chromatograms under UV light at a wavelength of 366 nm at UV lamp CAMAG 4 (Switzerland) and evaporating solvent traces, the plates were soaked in potato-glucose nutrient medium, then a propagule suspension of test fungus (*F. oxysporum* var. *orthoceras* BZR 6) was applied and placed in a moist chamber at 28.0 °C for 48 h. The localization of the active components was identified by the formation of zones of the absence or growth inhibition of the fungus, and the level of their anti-fungal activity by their size (Sherma and Fried, 2003).

2.4. Statistics

Statistical data processing was performed by standard methods using MS Excel and ANOVA program for Windows. All data were expressed as mean from triplicate samples ± standard deviation. Duncan test was used and differences were considered statistically significant at $p < 0.05$ level.

3. Results and discussion

Experiments showed that the maximum number of cells ($1.6–1.7 \times 10^9$ CFU/ml) in the cultivation of *B. subtilis* strain BZR 336 g was observed at a temperature of 20.0–25.0 °C. The highest titer of the liquid culture of the *B. subtilis* BZR 517 strain (8.9×10^8 CFU/ml) was obtained at a temperature of 30.0 °C. A comparative study of the antibiotic activity of the culture fluid of the studied strains in relation to the test culture of *F. oxysporum* var. *orthoceras* BZR 6 showed that the cultivation temperature significantly affects the antibiotic activity (Fig. 1).

In the course of the research it was noted that intensive accumulation of antifungal substances in the medium was recorded

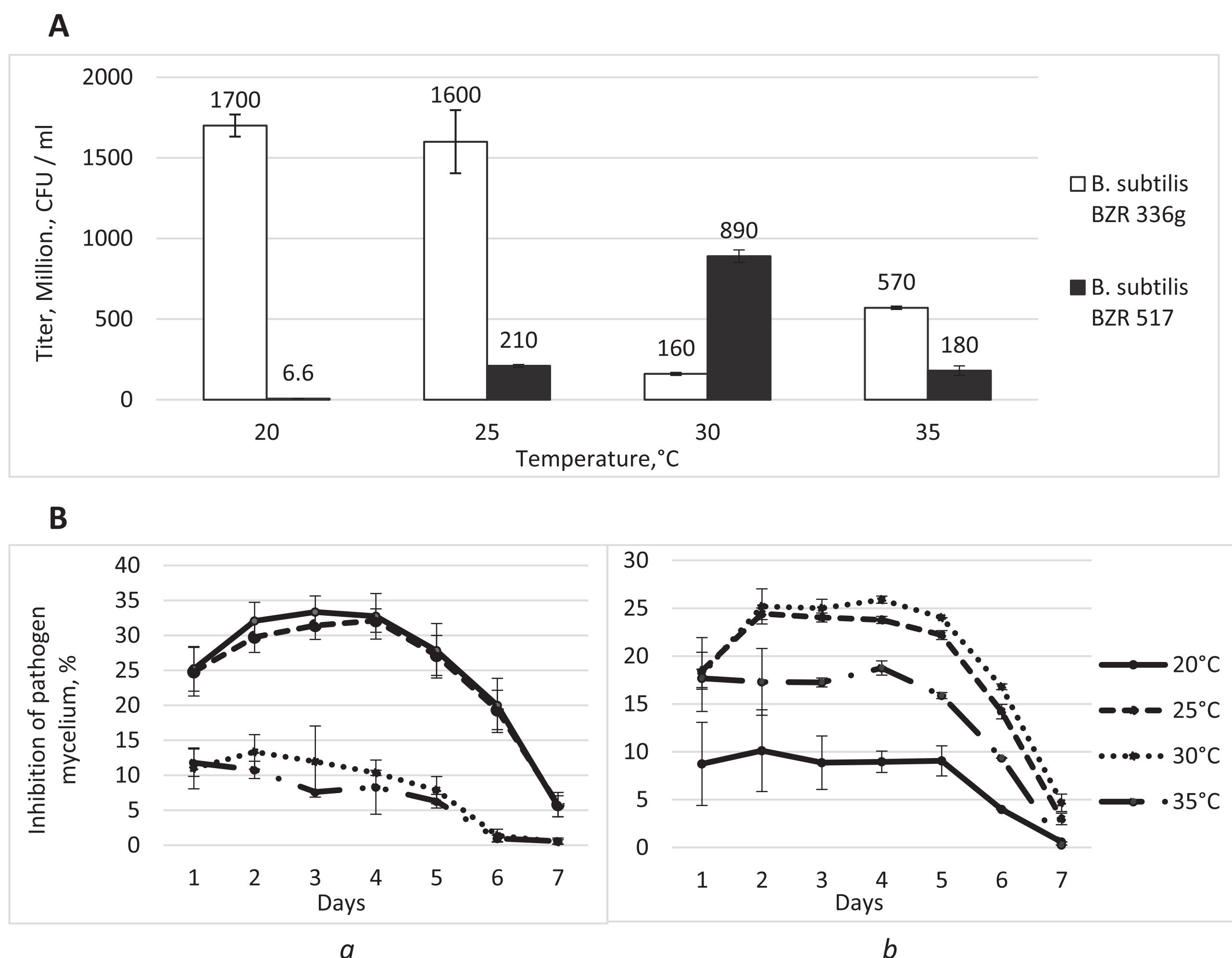

Fig. 1. (A) Growth of *B. subtilis* BZR336g and *B. subtilis* BZR517 strains during periodic cultivation. (B) Inhibition of growth of *F. oxysporum* var. *orthoceras BLR 6* depending on the temperature of periodic cultivation of the strains *B. subtilis* BZR336g – *a* and *B. subtilis* BZR 517 – *b*.

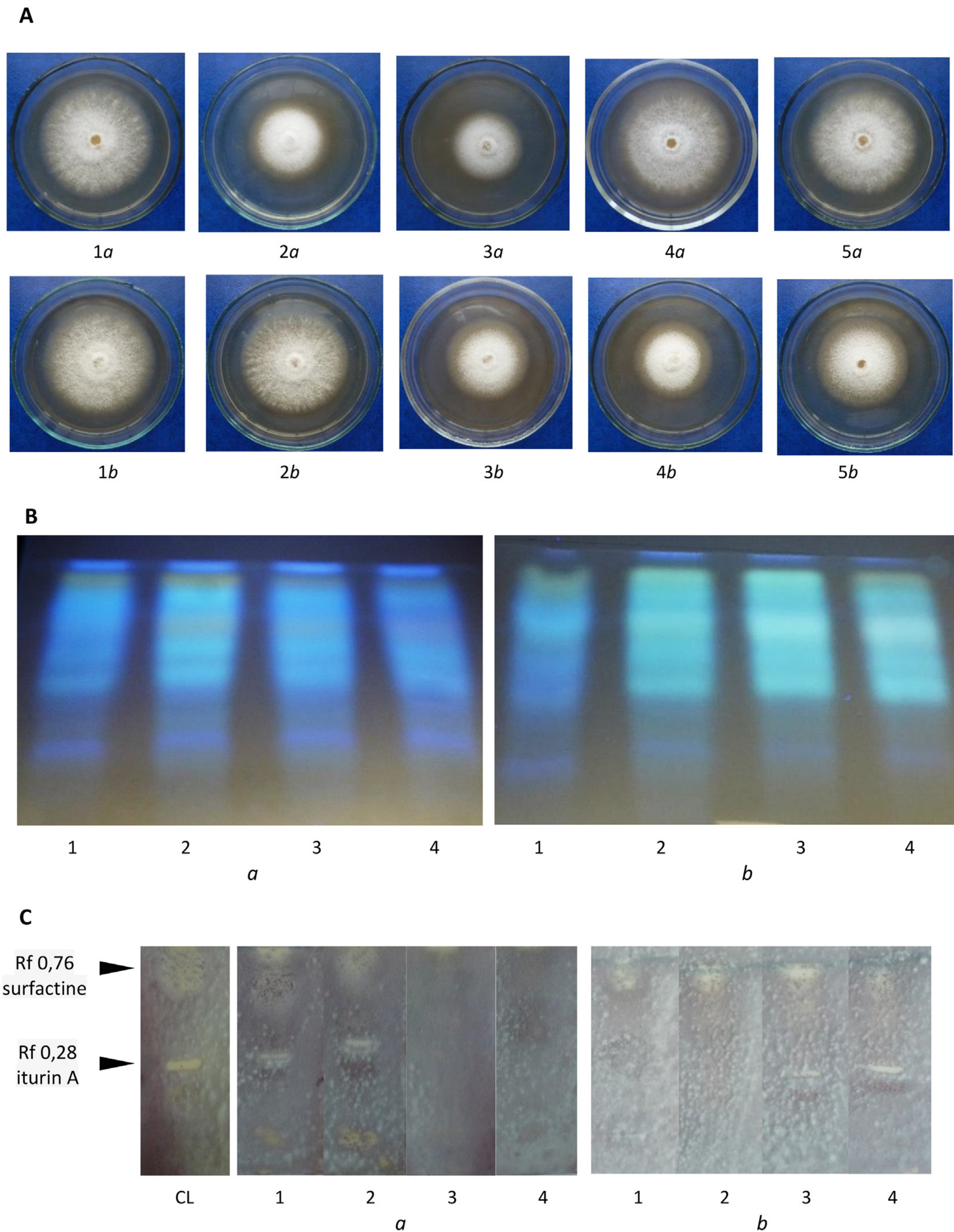

Fig. 2. Inhibition of growth of the *F. oxysporum* var. *orthoceras BZR 6* depending on the temperature of periodic cultivation of *B. subtilis* BZR336g strains - *a* and *B. subtilis* BZR517 strains – *b*. (A) Antibiotic activity depending on the temperature of periodic cultivation (1 – control; 2 – 20.0 °C; 3 – 25.0 °C; 4 – 30.0 °C; 5 – 35.0 °C). (B) Chromatograms of liquid culture on silica gel medium in UV 366 light (ethyl acetate - ethanol - water 40:15:15) (1–20.0 °C; 2–25.0 °C; 3–30.0 °C; 4 – 35.0 °C). (C) Bioautograms of commercial lipopeptides (CL) and liquid culture obtained at different temperatures of periodic cultivation (1–20.0 °C; 2–25.0 °C; 3–30.0 °C; 4–35.0 °C).

at 20.0–25.0 °C for *B. subtilis* BZR 336 g strain and at 25.0–30.0 °C for *B. subtilis* BZR 517 strain. Thus, for the *B. subtilis* BZR 336 g strain, the accumulation of antibiotic metabolites decreased with an increase in cultivation temperature in 3–4 times. For the strain *B. subtilis* BZR 517 temperature decrease was a stress factor, which led to a significant decrease in antibiotic activity (up to 10.0%) against *F. oxysporum* var. *orthoceras* BZR 6.

Both studied strains were found to produce a large number of compounds, differing both in mobility and in the nature of luminescence in UV366 light.

Simple profiling by upward thin layer chromatography is an excellent bioanalytical technique that, when using standard commercial lipopeptides, assesses the ability of a bacterial strain to synthesize antifungal metabolites (Fig. 2).

When comparing the obtained lipopeptide profiles with commercial surfactin (Rf 0.76) and iturinA (Rf 0.28), it was found that both strains produce both the first and the second lipopeptide. Visual assessment allows us to conclude that the *B. subtilis* BZR 336g strain accumulates surfactin and iturinA more than the *B. subtilis* BZR 517 strain.

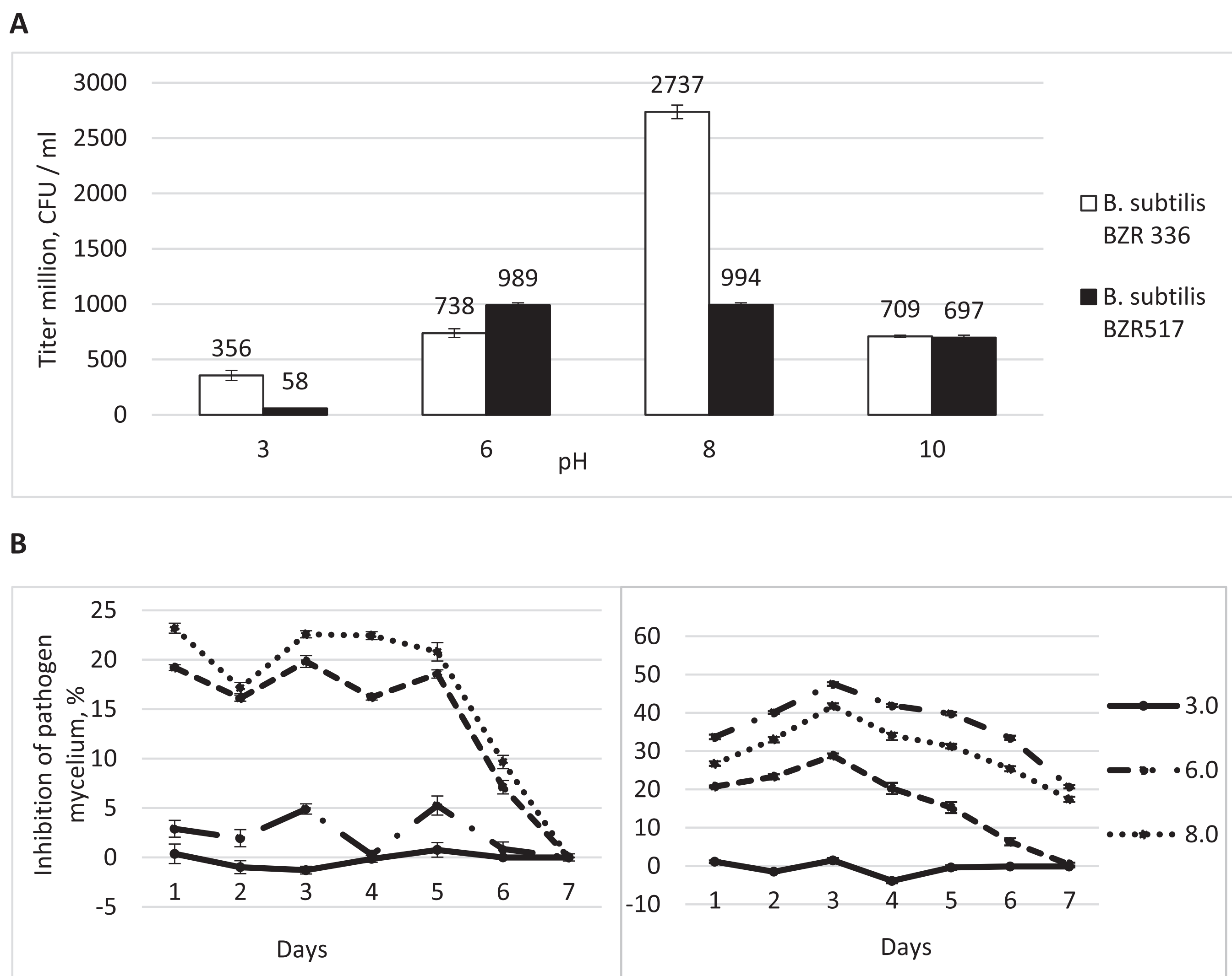

Fig. 3. (A) Growth of *B. subtilis* BZR336g and *B. subtilis* BZR517 strains in the process of periodic cultivation. (B) Inhibition of growth of *F. oxysporum* var. *orthoceras BZR 6* depending on the acidity of the medium of periodic cultivation of the *B. subtilis* BZR336g strain – *a* and *B. subtilis* BZR 517 strain – *b*.

The chromatograms and bioautograms analysis allows us to report that surfactin and IturinA at a temperature of 20.0–25.0 °C - for *B. subtilis* BZR 336g strain and 30.0–35.0 °C - for *B. subtilis* BZR 517 strain are best produced by bacteria.

It was established that the highest antibiotic activity corresponding to the maximum density of cells in liquid culture was observed at the medium pH 8.0 for the *B. subtilis* BZR 336 g strain (2.7×10^9 CFU ml) and at the medium pH 6.0–8.0 for the *B. subtilis* BZR 517 strain (1.0×10^9 CFU ml). It was noted that the *B. subtilis* BZR 517 strain exhibited high antibiotic activity in a greater pH range than the *B. subtilis* BZR 336 g strain (Fig. 3).

The change in the acidity of the nutrient medium influences the qualitative and quantitative composition (the glow pattern under UV light UV366) of metabolites and it is individual for each strain.

The results of chromatographic and bioautographic studies of the studied metabolites antibiotic activity depending on the acidity of the nutrient medium are presented in Fig. 4.

Analysis of chromatograms under UV light 366 allowed us to detect differences in antifungal metabolites in terms of luminescence, as well as in chromatographic mobility, depending on the pH of the cultivation medium. A bioautographic study of the culture fluids of the studied strains showed that the most active antifungal compounds are formed by the *B. subtilis* BZR 336 g strain at pH 8 and by *B. subtilis* BZR 517 strain with the acidity index of 8–10. A bioautographic study of the culture fluids of the studied strains showed that pH 8.0 is most optimal for the synthesis of surfactin and iturin A by *B. subtilis* BZR 336g, and the acidity index is 8.0–10.0 for *B. subtilis* BZR 517 strain. It should be noted that with a higher acidity of the nutrient medium, iturinA is produced in trace amounts or is not synthesized at all.

In the course of the research we revealed the influence of temperature and acidity on the antifungal metabolites synthesis by two *B. subtilis* strains. According to numerous studies, *B. subtilis* bacteria are able to grow and synthesize antifungal metabolites in the temperature range from 25 to 37 °C and at pH values from 5 to 9 (Chen et al., 2015). Thus, for *B. subtilis* BBG208strain, the highest yield of fengicin was recorded at 30 °C and pH 7. A shift in temperature to 25 and 35 °C significantly reduced the fengicin synthesis (Yaseen et al., 2017). This pattern was noted for *B. subtilis* C9 strain, (Islam et al., 2012) and for *B. subtilis* KLP2015 strain (Meena et al., 2020). However, for the growth of *B. subtilis* MS21 strain, the optimum shifts toward 35 °C and pH 8 (Anjhana and Sasikala, 2017). Four *B. subtilis* strains with antagonistic activity against *Rhizoctonia solani* were able to grow at temperatures from 15 to 37 °C and in a wide pH range from 5 to 9. But the maximum number of cells was fixed at 22 °C and pH 9 (Mousivand et al.,

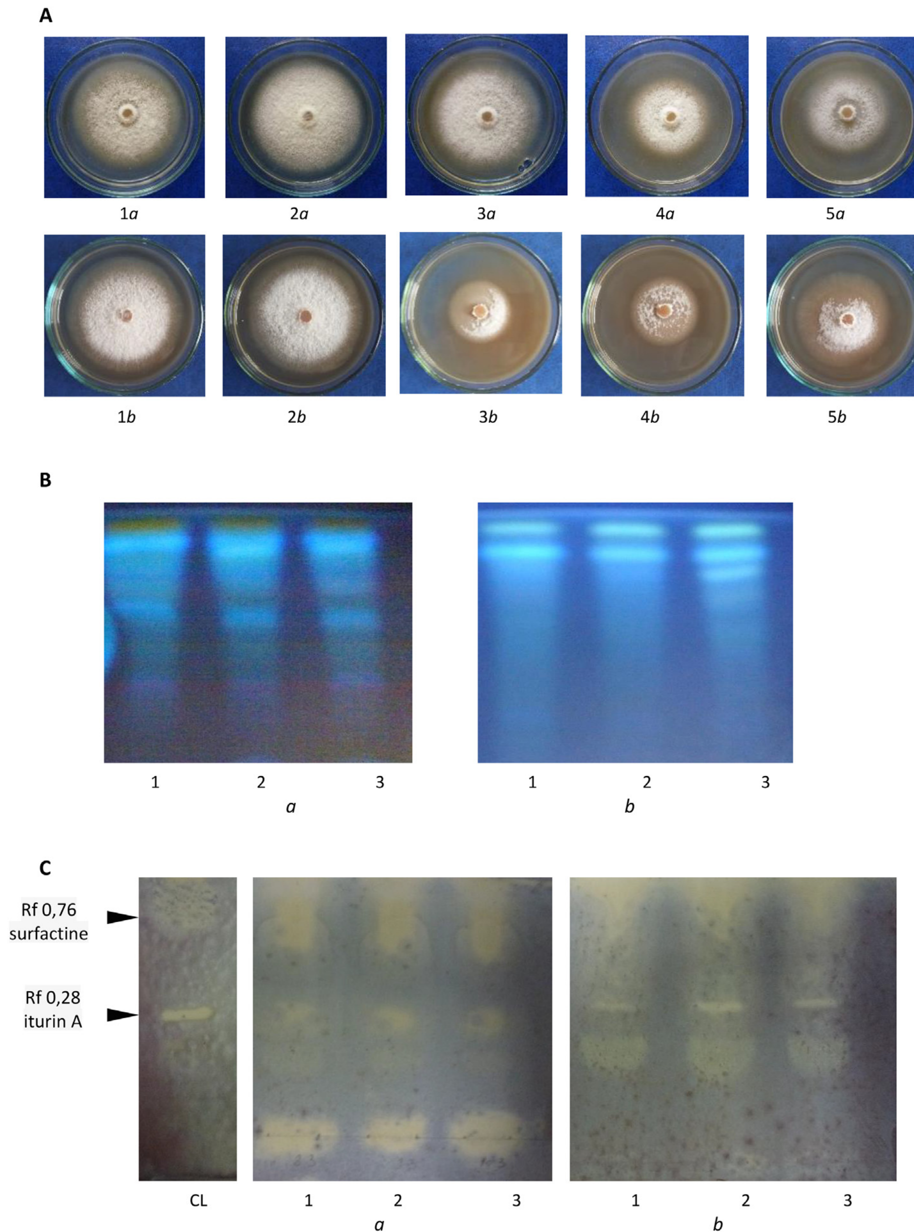

Fig. 4. Inhibition of growth of the *F. oxysporum* var. *orthoceras BZR 6* depending on the acidity of the medium of periodic cultivation of *B. subtilis* BZR336g strains – *a* and *B. subtilis* BZR517 strains – *b*. (A) Antibiotic activity depending on the temperature of periodic cultivation (1 – control; 2 – 3.0; 3 – 6.0; 4 – 8.0; 5 – 10.0). (B) Chromatograms of liquid culture on silica gel medium in UV 366 light (ethyl acetate - ethanol - water 40:15:15) (1 – 3.0; 2 – 6.0; 3 – 8.0; 4 – 10.0). (C) Bioautograms of commercial lipopeptides (CL) and liquid culture obtained at different pH of periodic cultivation (1 – 3.0; 2 – 6.0; 3 – 8.0; 4 – 10.0).

2012). This difference is due to strain differences. Thus, for each new strain, it is necessary to determine individual cultivation conditions, which is confirmed in our studies.

The criteria that are necessary for an effective fungicidal preparation is antifungal activity against phytopathogens in combination with a high number of colony forming units. These criteria allow antagonist bacteria to efficiently introduce themselves into the ecosystem and provide a protective effect. Therefore, the difference in sensitivity to different factors allows us to obtain such parameters that will ensure a high-quality preparation. Moreover, by developing fungicides based on microorganisms sensitive to various factors, we can significantly expand the range of commercial products suitable for application in various agro-climatic zones in future.

The obtained data on the optimal cultivation conditions of two promising *B.subtilis* strains, which contribute not only to the maximum accumulation of bacterial cells, but also stimulate the secretion of fungitoxic lipopeptides, should be used in developing technologies for the production of effective biofungicides to protect crops against harmful fungal diseases pathogens.

4. Conclusions

Thus, our studies have shown that despite the fact that both strains belong to the species *B. subtilis*, cultivation parameters such as temperature and acidity of the medium affect the number of colony-forming units and the synthesis of their antifungal metabolites in different ways. It was found that for the *B. subtilis* BZR 336 g strain the optimum cultivation temperature was 25.0 °C, the optimum acidity of the medium was 8.0, and for the *B. subtilis* BZR 517 strain the optimum cultivation temperature was 30.0 °C, and the

optimum acidity of the medium was 7.0–8.0. Namely, under these conditions the largest number of colony-forming units for two *B. subtilis* strains was observed together with high antibiotic activity against the fungus *F. oxyporum* var. *orthoceras BZR 6*.

The data obtained in this research can be used in the development of the technology for the production of effective biofungicides to protect crops against *Fusarium* pathogens.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abada, E.A., El-Hendawy, H.H., Osman, M.E., Hafez, M.A., 2014. Antimicrobial activity of *Bacillus circulans* isolated from rhizosphere of Medicago sativa. Life Sci. J. 11 (8), 711–719.

Anjhana, V.R., Sasikala, S.L., 2017. Isolation, screening and growth optimization of antagonistic *Bacillus subtilis* MS21 from Thengapattanam estuary against plant fungal pathogens. Int. J. Adv. Res. Biol. Sci. 4 (12), 15–26. https://doi.org/10.22192/ijarbs.2017.04.12.003.

Asaturova, A.M., Homyak, A.I., Tomashevich, N.S., Pavlova, M.D., Zhevnova, N.A., Dubyaga, V.M., Kozitsin, A. Ye., Sidorova, T.M., Nadykta, V.D., Ismailov, V.Ya., 2015. Conditions for the cultivation of new *Bacillus* bacteria being micro bioproduct producers. J. Pure Appl. Microbiol. 9 (4), 2797–2804.

Backer, R., Rokem, J.S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., Subramanian, S., Smith, D.L., 2018. Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Front. Plant. Sci. 9, 1473. https://doi.org/10.3389/fpls.2018.01473.

Bartolini, M., Cogliati, S., Vileta, D., Bauman, C., Ramirez, W., Grau, R., 2019. Stressresponsive alternative sigma factor SigB plays a positive role in the antifungal proficiency of *Bacillus subtilis*. Appl Environ Microbiol 85, e00178–e219. https://doi.org/10.1128/AEM.00178-19.

Chen, W.-C. Juang, R.-S., Wei, Y.-H., 2015 Applications of a lipopeptide biosurfactant, surfactin, produced by microorganisms. Biochem. Engin. J. 103, 158-169. 10.1016 / j.bej.2015.07.009.

Debois, D., Fernandes, O., Franzil, L., Jaordan, E., Brognier, A. De, Willems, L., Clement, C., Dorey, S., 2015. Plant polysaccharides initiate underground crosstalk with bacilli by inducing synthesis of the immunogenic lipopeptide surfactin. FEMS Microbiol. Rev. 10, 1758–2229. https://doi.org/10.1111/1758-2229.12286.

Egorov, N.S., 2004. Fundamentals of the theory of antibiotics (Vol. 528). Moscow State University: The science, Moscow.

Eschlbeck, E., Bauer, S.A.W., Kulozik, U., 2017. Effect of cultivation pH on the surface hydrophobicity of *Bacillus subtilis* spores. AMB Expr. 7, 157. https://doi.org/10.1186/s13568-017-0458-2.

Farace, G., Fernandes, O., Jacquens, L., Coutte, F., Krier, F., Jacques, Ph., Clement, Ch., Barka, E.A., Jacquard, C., Dorey, S., 2015. Cyclic lipopeptides from *Bacillus subtilis* activate distinct patterns of defence responses in grapevine. Molecul. Plant. Pathol. 16 (2), 177–187. https://doi.org/10.1111/mpp.12170.

Glare, T.R., Gwynn, R.L., Moran-Diez, M.E., 2016. Development of biopesticides and future opportunities. In: Glare, T.R., Moran-Diez, M.E. (Eds.), Microbial-Based Biopesticides. Humana Press, New York, pp. 211–221.

Harwood, C.R., Mouillon, J.M., Pohl, S., Arnau, J., 2018. Secondary metabolity of industrially impotant members of the *Bacillus subtillis* group. FEMS Microbiol. Rev. 42 (6), 721–738. https://doi.org/10.1093/femsre/fuy028.

Hashem, A., Tabassum, B., Fathi Abd Allah, E., 2019. *Bacillus subtilis*: A plant-growth promoting rhizobacterium that also impacts biotic stress. Saud. J. Biol. Sci. 26 (6), 1291–1297. https://doi.org/10.1016/j.sjbs.2019.05.004.

Islam, M.R., Jeong, Y.T., Lee, Y.S., Song, C.H., 2012. Isolation and identification of antifungal compounds from *Bacillus subtilis* C9 inhibiting the growth of plant pathogenic fungi. Mycobiol. 40 (1), 59–65. https://doi.org/10.5941/MYCO.2012.40.1.059.

Kumar, S., Singh, A., 2015. Biopesticides: present status and the future prospects. J. Fertil Pestic. 6 (2), 100–129. https://doi.org/10.4172/jbfbp.1000e129.

Li, X., Zhang, Y., Wei, Z., Guan, Z., Cai, Y., Liao, X., 2016. Antifungal activity of isolated Bacillus amyloliquefaciens SYBC H47 for the biocontrol of peach gummosis. PloS one. 11 (9). https://doi.org/10.1371/journal.pone.0162125.

Li, Y., Héloir, M.-C., Zhang, X., Geissler, M., Trouvelot, S., Jacquen, L., Henkel, M., Xin, I., Fang, X., Wang, Q., Adrian, M., 2019. Surfactin and fengycin contribute to the protection of a *Bacillus subtilis* strain against grape downy mildew by both direct effect and defence stimulation. Mol. Plant. Pathol. 20 (8), 1037–1050. https://doi.org/10.1111/mpp.12809.

Lirong, Y., Quana, X., Xuea, B., Goodwinb, P.H., Lua, S., Wanga, J., Dua, W., Wua, C., 2015. Isolation and identification of *Bacillus subtilis* strain YB-05 and its antifungal substances showing antagonism against *Gaeumannomyces graminis* var. *tritici*. Biol. Control. 85, 52–58. https://doi.org/10.1016/j.biocontrol.2014.12.010.

Meena, K.R., Sharma, A., Kumar, R., Kanwar, S.S., 2020. Two factor at a time approach by response surface methodology to aggrandize the *Bacillus subtilis* KLP2015 surfactin lipopeptide to use as antifungal agent. J. King Saud Univ.-Sci. 32, 337–348. https://doi.org/10.1016/j.jksus.2018.05.025.

Mnif, I., Grau-Campistany, A., Coronel-León, J., Hammami, I., Triki, M.A., Manresa, A., Ghribi, D., 2016. Purification and identification of *Bacillus subtilis* SPB1 lipopeptide biosurfactant exhibiting antifungal activity against *Rhizoctonia bataticola* and *Rhizoctonia solani*. Environ. Sci. Pollut. Res. 23 (7), 6690–6699. https://doi.org/10.1007/s11356-015-5826-3.

Montealegre, J.R., Reyes, R., Perez, L.M., 2003. Selection of antagonists to be used in biological control of *Rhizoctonia solani* in tomato. Electron. J. Biotechnol. 6 (2), 116–127. https://doi.org/10.2225/vol6-issue2-fulltext-8.

Mousivand, M., Jouzani, G.S., Monazah, M., Kowsari, M., 2012. Characterization and antagonistic potential of some native biofilm-forming and surfactant-producing *Bacillus subtilis* strains against six pathotypes of *Rhizoctonia solani*. J. Plant Pathol. 94 (1), 171–180.

Nawaz, H.H., Rajaofera, M.N., He, Q., Anam, U., Lin, C., Miao, W., 2018. Evaluation of antifungal metabolites activity from *Bacillus licheniformis* OE-04 against Colletotrichum gossypii. Pesticide Biochem. Physiol. 146, 33–42. https://doi.org/10.1016/j.pestbp.2018.02.007.

Netrusov, A.I., 2005. Workshop on microbiology (Vol. 608). Academy, Moscow.

Sherma, J., Fried, B., 2003. Handbook of thin-layer chromatography. Marcel Dekker, New York.

Toral, L., Rodríguez, M., Bejar, V., Sampedro, I., 2018. Antifungal activity of lipopeptides from Bacillus XT1 CECT 8661 against *Botrytis cinerea*. Front Microbiol. 9, 1315. https://doi.org/10.3389/fmicb.2018.01315.

Torres, M.J., Brandan, C.P., Sabaté, D.C., Petroselli, G., Erra-Balsells, R., Audisio, M.C., 2017. Biological activity of the lipopeptide-producing *Bacillus amyloliquefaciens* PGPBacCA1 on common vulgaris L. pathogens. Biol. Control. 105, 93–99. https://doi.org/10.1016/j.biocontrol.2016.12.001.

Tumbarski, Y., Petkov, E., Denkova, Z., 2015. Study on the influence of the cultural conditions and the composition of the culture medium on the antimicrobial activity of *Bacillus methylotrophicus* BM47 against some fungal phytopathogens. J Glob. Biosci. 4 (8), 2990–2996.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., Nasrulhaq, B.A., 2016. Role of plant growth promoting rhizobacteria in agricultural sustainability – A Review. Molec. 21 (573), 1–17. https://doi.org/10.3390/molecules21050573.

Volova, T., Kiselev, E., Vinogradova, O., Nikolaeva, E., Chistyakov, A., Sukovatiy, A., Shishatskaya, E., 2014. A glucose-utilizing strain, Cupriavidus euthrophus B-10646: growth kinetics, characterization and synthesis of multicomponent phas. PLoS ONE. 9 (2), 1–15. https://doi.org/10.1371/journal.pone.0087551.

Wang, T., Liang, Y., Wu, M., Chen, Z., Lin, J., Yang, L., 2015. Natural products from *Bacillus subtilis* with antimicrobial properties. Chin. J. Chem. Eng. 23 (4), 744–754. https://doi.org/10.1016/j.cjche.2014.05.020.

Yaseen, Y., Gancel, F., Béchet, M., Drider, D., Jacques, P., 2017. Study of the correlation between fengycin promoter expression and its production by *Bacillus subtilis* under different culture conditions and the impact on surfactin production. Arch Microbiol. 199, 1371–1382. https://doi.org/10.1007/s00203-017-1406-x.

Yi, Y.J., Li, Y.S., Xia, B., Li, W.P., Pang, L., Tong, Z.D., 2015. Optimization of medium composition and culture conditions for antifungal activity of a tomato endophytic bacterium. Biol. Control. 82, 69–75. https://doi.org/10.1016/j.biocontrol.2014.12.009.