

# The study of the antifungal activity of the *Bacillus subtilis* BZR 336g strain under the conditions of periodic cultivation with the addition of citric acid, corn extract and some microelements

Anzhela Asaturova, Evgeny Gyrnets, Valeria Allakhverdian\*, Mikhail Astakhov, and Ksenia Saenko

All-Russian Research Institute of Biological Plant Protection, Krasnodar-39, 350039, Russia

**Abstract.** We studied the antifungal activity of the *Bacillus subtilis* BZR 336g strain against the test culture of the fungus *Fusarium oxysporum* var. *orthoceras* App. et Wr. BZR 6, depending on the addition of citric acid crystalline hydrate, a microelements solution and corn extract to the liquid nutrient medium. It was found that citric acid at a concentration of 15 g/l improves the bioavailability of microelements and increases antifungal activity. Corn extract and microelements without the formation of a chelate form with citric acid do not affect the fungicidal properties of *B. subtilis* BZR 336g. However, the corn extract at a concentration of 3 g / l increases the titer of bacteria in the liquid culture from  $2 \pm 0.1 \times 10^8$  to  $1 \pm 0.08 \times 10^8$  CFU/ml. The combined use of the studied components allowed us to achieve a significant increase in the antifungal activity of *B. subtilis* BZR 336g by 3.1 times. At the same time, the effect of synergism in their complex interaction was noted, which is probably due to a qualitative and quantitative change in the composition of *B. subtilis* BZR 336g antifungal metabolites.

## 1 Introduction

Currently, due to the rapidly developing industry for the production and processing of organic products, the requirements for the biological products are becoming increasingly demanding. In this regard, increasing the efficiency and stability of biological products is an important task of biotechnological production.

Bacillus bacteria, on the basis of which many biological preparations are produced, have great agricultural potential, particularly due to the production of lipopeptides with high fungicidal, insecticidal and nematicidal activity [1].

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\*Corresponding author: [lera\\_arm@mail.ru](mailto:lera_arm@mail.ru)

Antifungal activity was found mainly in three families of lipopeptides: surfactins, iturins, and fengicins. These compounds are amphiphilic in nature, due to which they are able to penetrate into the cells and destroy the membrane structures of fungal pathogens [2].

The biosynthesis of lipopeptide compounds in *Bacillus* bacteria is a part of a complex metabolic network. Thus, fengicin, iturin, and surfactin are biosynthesized by many non-ribosomal peptide synthetases (NPS synthetases) [3-6]. Many of these enzymes are known to be complex. They include coenzymes of various nature, metal ions and vitamins in particular [7].

Therefore, the synthesis of *B. subtilis* fungicidal substances can be significantly increased by adding various mineral salts and sources of vitamins. That's why, rich complex component media can act as nutrient media for growing microorganisms producing antibiotic substances [8, 9].

The team of researchers Kim et al. [10] studied the dependence of the production of the iturin, fengicin and surfactin lipopeptides with the addition of various metal ions ( $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cs}^+$ ,  $\text{Ni}^+$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ) in M9 broth. As a result, the production of lipopeptides was increased by 2.6 times by adding  $10^{-6}$  M  $\text{Mn}^{2+}$  ion.

However, it is necessary to take into account the possible negative effect of microelements. The introduction of  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$  negatively affected the production of iturin A. Iron nanoparticles showed significant toxicity against *B. subtilis* 3610 and *B. thuringiensis* 407 [11, 12].

Another objective of biotechnology is to increase the bioavailability of microelements found in insoluble or sparingly soluble compounds. One way to solve it is to add substances that increase the solubility of microelements to liquid nutrient media. So, it is known that citric acid forms water-soluble chelate complexes with many metal ions [13].

In the biotechnology industry, various by-products are used as growth stimulators and a source of vitamins. For *B. subtilis*, corn extract is often used because it is a source of B vitamins, biotin in particular [14]. This component is known to have the positive effect on the production of lipopeptide biosurfactants [15].

The antifungal activity of *B. subtilis* also depends on the titer of bacterial cells, which is caused, among other factors, by the ability to sporulate. A group of researchers from the Institute of Experimental Biology (Portugal) tested a mixture of several vitamins, and separately thiamine, as the most important vitamin for sporulation. As a result, the spore yield obtained using 10 mg/l thiamine was similar to the result obtained using the vitamin complex [16].

Thus, the aim of our study is to find ways to increase the antifungal activity of the biocontrol *B. subtilis* BZR 336g strain by adding mineral salts and sources of vitamins, as well as improving the bioavailability of medium components under conditions of periodic cultivation.

## 2 Materials and methods

The objects of the study were *B. subtilis* BZR 336g strain, the basis of the microbiological biofungicide developed at the ARRIBPP, and the test culture of the fungus *Fusarium oxysporum* var. *orthoceras* App. et Wr. BZR 6 from the Bioresource collection of the FSBSI ARRIBPP "The State Collection of Entomocariphages and Microorganisms".

For the research we used the material and technical base of the unique scientific installation "Technological line for obtaining microbiological plant protection products of a new generation" (<http://ckp-rf.ru/usu/671367/>).

As a standard we used the original nutrient medium (ONM) developed at the FSBSI ARRIBPP – a semi-synthetic medium containing organic carbon sources, a non-organic source of nitrogen, and mineral salts. Sources of carbon and nitrogen, as well as the

concentration of the introduced components of ONM as elements of the technology for the production of new biological products are the objects of the trade secret of the FSBSI ARRIBPP [17]. The studied components were added to the ONM to determine their effect on the antifungal activity of *B. subtilis* BZR 336g.

**To determine the optimal concentration of citric acid (CA)**, CA crystalline hydrate was added to the ONM in increments of 0.5 g until the residuum was completely dissolved. Weighed portions were measured using an analytical scale Adventurer Pro, AV4102C (USA). The pH of the medium was adjusted to 6 by adding a 20 % NaOH solution using a Sartorius PB-11 pH meter (Germany).

**The preparation of a microelement (ME) solution** was carried out according to the Thermo Fisher Scientific guidelines for *Pichia pastoris* (due to the most complete ME coverage). The composition of the ME solution (g/l): CuSO<sub>4</sub> × 5 H<sub>2</sub>O (3.84); KI (0.08); MnSO<sub>4</sub> × 5 H<sub>2</sub>O (2.68); Na<sub>2</sub>MoO<sub>4</sub> × 2 H<sub>2</sub>O (0.23); H<sub>3</sub>BO<sub>3</sub> (0.02); CoCl<sub>2</sub> × 6 H<sub>2</sub>O (0.92); ZnSO<sub>4</sub> × 7 H<sub>2</sub>O (42.2); FeSO<sub>4</sub> × 7 H<sub>2</sub>O (65.0). The pH of the ME solution was reduced to 2.4 by the addition of CA until the residuum was completely dissolved and then added to ONM at a concentration of 4.4 ml/l. The pH of the resulting medium was adjusted to 6.0 with a 20 % NaOH solution.

**To determine the optimal concentration of corn extract (CE)**, we applied it in the range from 2 to 7 g/l in increments of 1 g/l. The minimum concentration of CE is determined as 2 g/l due to the fact that in the formulations of liquid culture media using CE its concentration varies between 2-5 g / l [14, 15].

After the combined use of CA at a concentration of 15 g/l, a solution of ME at a concentration of 4.4 g/l and CE at a concentration of 3 g/l, the pH of the medium was also adjusted to 6 by the addition of a 20 % NaOH solution.

Parent and liquid culture (LC) based on the *B. subtilis* BZR 336g strain was obtained in the incubator shakers New Brunswick Scientific Excella E25 (USA) under conditions of periodic cultivation. Cultivation was carried out in 1000 ml flasks (medium volume 300 ml) at 180 rpm, + 25.0 °C and pH 6.0 for 48 hours. The experiment was repeated three times.

The effect of CA, ME, and CE on the antifungal activity of the strain-producer LC of a biological product was studied in comparison with the standard. At the same time, indicators such as bacterium titer and antifungal activity were assessed.

The number of colony forming units (CFU) was determined at the end of the cultivation in 1 ml of LC by the Koch method (in-depth method) using the system for automatic colony counting Color Qcount, Spiral Biotech (USA) [18].

The antifungal activity of *B. subtilis* BZR 336g strain was determined by the dual culture method [19]. As a test culture, we used *F. oxysporum* var. *orthoceras* BZR 6. Cultivation was carried out at a temperature of + 23 ° C. Recording was carried out on the 5th, 10th, 15th, 20th day.

Antifungal activity was calculated by the formula [20]:

$$N = \left(1 - \frac{d_n}{D}\right) \times 100, \tag{1}$$

where N – test culture inhibition, %  
 D – the diameter of the test culture colony in the control, mm,  
 $d_n$  – the diameter of the test culture colony in the experimental option, mm

To compare the experimental options and the standard we used the formula:

$$\Delta N = N_n - N_{\text{standard}}, \tag{2}$$

where ΔN – difference in ability to inhibit test culture, %  
 $N_n$  – experimental option inhibition, %  
 $N_{\text{standard}}$  – standard inhibition, %

Statistical data processing was performed using standard computer programs, as well as STATISTICA (v. 12.6).

### 3 Results and discussion

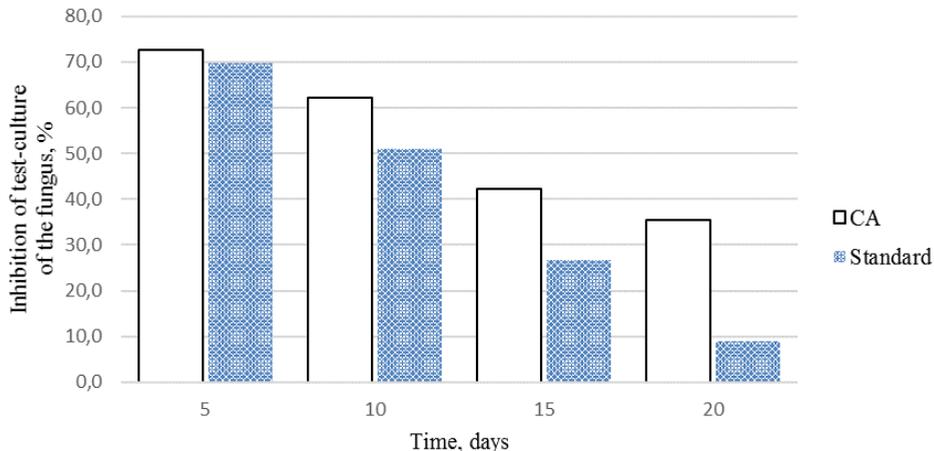
#### 3.1 The study of the *B. subtilis* BZR 336g antifungal activity with the addition of CA

The composition of OPS includes non-organic components containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions. These compounds have low solubility and form a mineral residuum.

As a result of the data analysis on the dissolution of mineral residuum, it was found that the optimal amount of the applied monohydrate of CA is 15 g/l. At this concentration, the pH of the medium decreases to 2.4. Bringing the pH of the medium to 6.0 does not lead to the residuum formation again. This fact indicates that the dissolution of salts is caused not by the pH level of the medium, but by the formation of chelate complexes with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions.

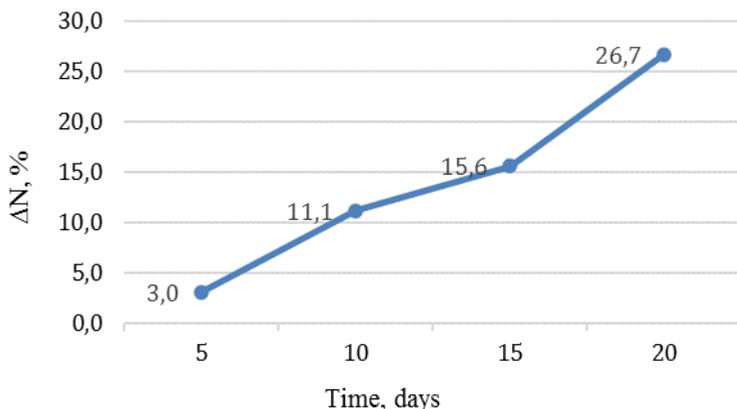
It was found that the bacterium titer in the variant and standard did not differ significantly ( $3.6 \pm 0.3 \times 10^8$  and  $5 \pm 0.8 \times 10^8$  CFU/ml, respectively). Thus, despite a rather high concentration, CA does not have any significant effect on the number of CFU in LC.

Moreover, it was found that CA improves the bioavailability of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  salts, thereby increasing the antifungal activity of LC. Significant differences are observed from the 10th day of cultivation (Fig. 1).



**Fig. 1.** The inhibition degree of *F. oxysporum* var. *orthoceras* BZR 6 by *B. subtilis* BZR 336g strain in the experimental option and the standard depending on the time of incubation

A linear increase in the positive difference in inhibition indicates a gradual decrease in antifungal activity in the standard and its preservation in the variant with the addition of CA (Fig. 2).

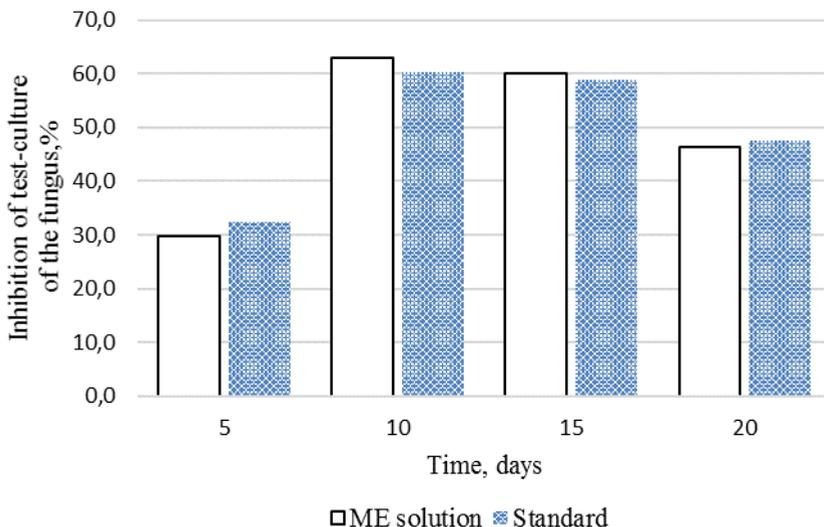


**Fig. 2.** The difference in the inhibition degree of *F. oxysporum* var. *orthoceras* BZR 6 by *B. subtilis* BZR 336g strain in the experimental option and the standard depending on the time of incubation

Thus, the use of CA as a substrate for dissolving the mineral residuum of the ONM makes it possible to significantly prolong the antifungal effect of the studied LC based on the *B. subtilis* BZR 336g strain.

**3.2 The study of the *B. subtilis* BZR 336g antifungal activity with the addition of ME**

We did not prove a reliable effect of ME on the values of the bacterial titer of LC and antifungal activity of the *B. subtilis* BZR 336g strain, which is confirmed by the results of studies using dual culture methods (Fig. 3).



**Fig. 3.** The inhibition degree of *F. oxysporum* var. *orthoceras* BZR 6 by *B. subtilis* BZR 336g strain in the experimental option and the standard depending on the time of incubation

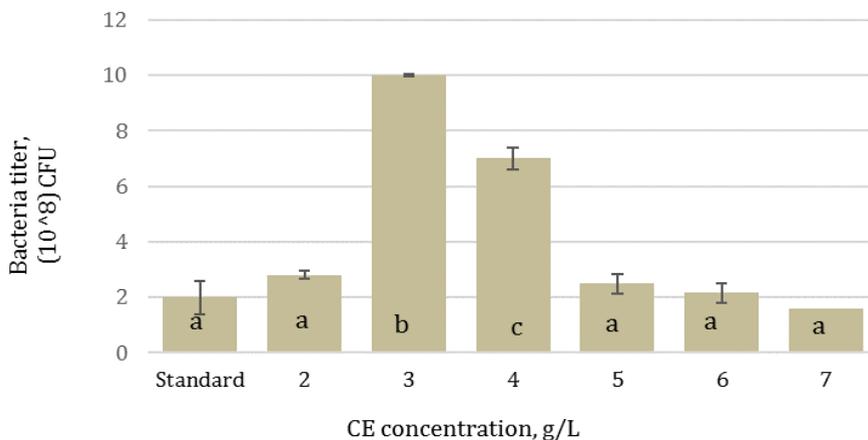
There is no effect of ME on the antifungal activity of the *B. subtilis* BZR 336g strain due to their low bioavailability in LC. So, in the working solution, the complex of salts and

acids, which include microelements, are in soluble form due to a pH of 2.4. However, when the solution is added to the LC, where the pH is 6.0, the compounds containing ME again turn into an insoluble or sparingly soluble form.

The obtained results allow us to test reasonably the combined use of CA monohydrate and ME solution.

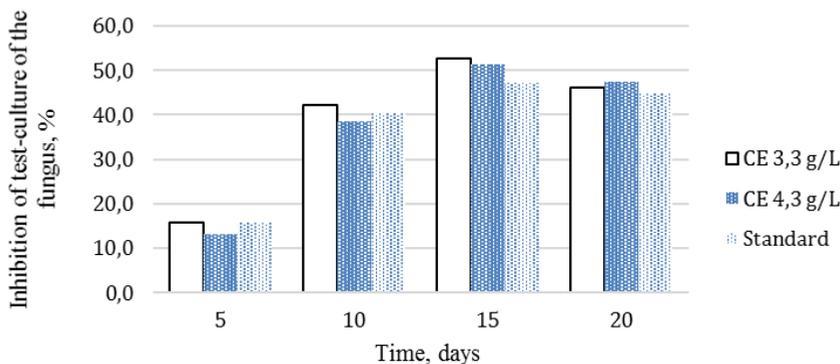
### 3.3 The study of the *B. subtilis* BZR 336g antifungal activity with the addition of CE

During the study we found that the addition of CE in concentrations of 3 and 4 g/l significantly increases the bacterial titer by 5 and 3.5 times, respectively (Fig. 4).



**Fig. 4.** The effect of CE on the titer of LC based on *B. subtilis* BZR 336g. The data included in the same group have no statistically significant differences according to the Duncan criterion at a 95 % level of significance

However, there were no significant differences in the antifungal activity of the strain when CE was added in the tested concentrations (Fig. 5).



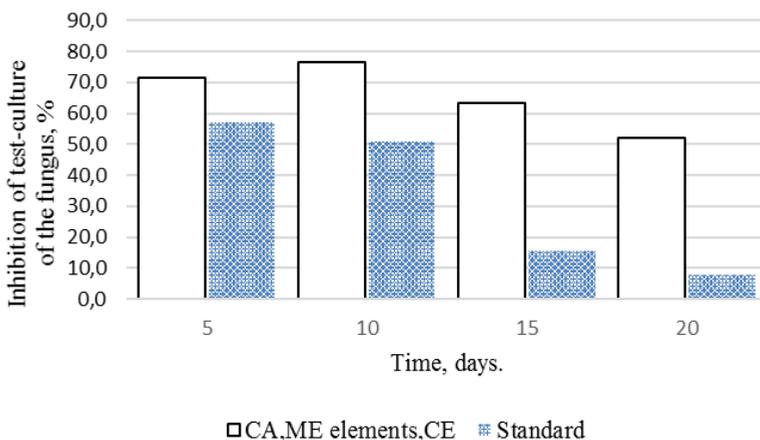
**Fig. 5.** The inhibition degree of *F. oxysporum* var. *orthoceras* BZR 6 by *B. subtilis* BZR 336g strain in the experimental option and the standard depending on the time of incubation

Thus, based on the obtained data, we can judge the positive effect of CE at a concentration of 3 g/l on the anabolism of cells of the *B. subtilis* BZR 336g strain-producer.

However, to significantly enhance the antifungal activity this component of the medium is not enough. This fact encourages us to carry out a study of CE in combination with other components.

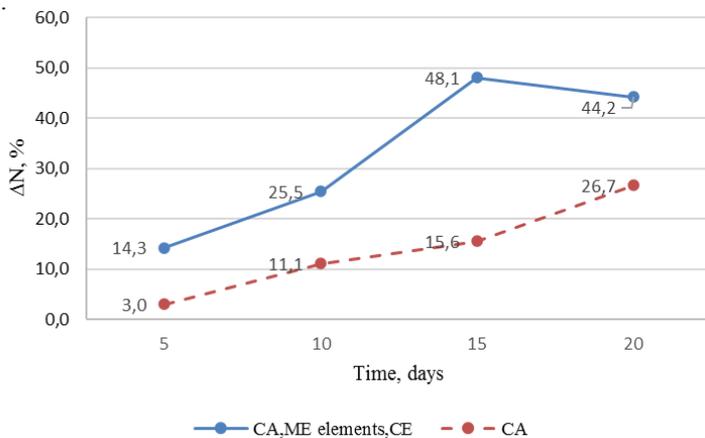
### 3.4 The study of the *B. subtilis* BZR 336g antifungal activity with the addition of a complex of CA, ME and CE

In the course of the study, we found that the combined effect of the components: CA at a concentration of 15 g/l, ME solution at a concentration of 4.4 ml/l and CE at a concentration of 3 g/l on the antifungal activity of *B. subtilis* BZR 336g strain is significantly higher than the effect of each component separately (Fig. 6). At the same time, there is no significant effect on the titer of bacteria ( $4.3 \pm 0.5 \times 10^8$  and  $4.0 \pm 1 \times 10^8$  CFU/ml in the experimental option and the standard, respectively).



**Fig. 6.** The inhibition degree of *F. oxysporum var. orthoceras* BZR 6 by *B. subtilis* BZR 336g strain in the experimental option and the standard depending on the time of incubation

The increase in  $\Delta N$  has a similar linear dependence with that for  $\Delta N$  CA. It was found that  $\Delta N$  of the complex of substrates on the 15th day is 3.1 times higher than  $\Delta N$  of the CA (Fig. 7).



**Fig. 7.** The difference in the inhibition degree of *F. oxysporum var. orthoceras* BZR 6 by *B. subtilis* BZR 336g strain in the experimental option and the standard depending on the time of incubation

The above mentioned indicates an improvement in the bioavailability of ME in the form of chelate complexes with CA. This factor probably influences the synthesis of NPS synthetases and, as a result, antifungal metabolites, which in turn increases the antifungal activity of *B. subtilis* BZR 336g strain.

## 4 Conclusions

As a result of the research, the antifungal activity and titer of LC bacteria based on the *B. subtilis* BZR 336g strain was studied depending on the addition of CA, ME, and CE to the ONM. It was determined that CA at a concentration of 15 g/l dissolves the mineral residuum of ONM and increases the antifungal activity of the strain. At the same time, there is no effect of the component on a significant change in the titer of bacteria.

The opposite effect is observed when CE is added in an amount of 3 g/l. At this concentration, the bacterial titer rises by 5 times, however, antifungal activity does not significantly change.

It was found that ME without improved solubility are in an inaccessible form for *B. subtilis* BZR 336g. The combined use of CA, ME and CE allows us to solve the problem of bioavailability of ME and significantly increase the antifungal activity of the studied strain LC. So the fungicidal effect in the experimental option on the 15th day is 3 times higher than the fungicidal effect of the standard. In this case, the effect of the combined use of CA, ME, and CE significantly exceeds the total effect of each component separately.

The obtained results allow us to reasonably recommend the combined use of CA, ME, CE as components of ONM to increase the antifungal activity of *B. subtilis* BZR 336g strain under conditions of periodic cultivation.

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