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Bacteriostatic Activity of *Janthinobacterium lividum* and Purified Violacein Fraction against *Clavibacter michiganensis*

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Abstract: *Clavibacter michiganensis* causes plant diseases and is included in the list of microorganisms subject to export control. *Janthinobacterium lividum* is capable of synthesizing a pigment with antagonistic potential. The purpose of the study was to evaluate the activity of *J. lividum* VKM B-3705D and the pigment fraction against *C. michiganensis* VKM Ac-1402. The results of spectrophotometric and nuclear magnetic resonance analysis showed that the pigment synthesized by the *J. lividum* VKM B-3705D corresponds to violacein. The *J. lividum* strain demonstrated potential bacteriostatic activity against *C. michiganensis* VKM Ac-1402 when both strains were co-cultured. Compared to the control (DMSO), the violacein solution suppressed the specific growth of *Clavibacter* by 57.7%. The mechanism of suppression of the growth of *Clavibacter* is discussed. One of the ways to suppress the growth of *C. michiganensis* may be the inhibition of key enzymes. Violacein inhibited the activity of adenosine triphosphatase (ATPase, EC 3.6.1.3) compared to the control (DMSO) by 23.2%. Thus, the current study of the bacteriostatic effect may be a decisive step towards the development of a plant protection product.

Keywords: *Clavibacter michiganensis*; *Janthinobacterium lividum*; bacteriostatic activity; violacein; intermicrobial interactions; ATP-ase; adenosine triphosphatase

Citation: Lyakhovchenko, N.S.; Efimova, V.A.; Seliverstov, E.S.; Anis'kov, A.A.; Solyanikova, I.P. Bacteriostatic Activity of *Janthinobacterium lividum* and Purified Violacein Fraction against *Clavibacter michiganensis*. *Processes* **2024**, *12*, 1116. https://doi.org/10.3390/pr12061116

Academic Editor: Adina Musuc

Received: 24 April 2024
Revised: 18 May 2024
Accepted: 24 May 2024
Published: 29 May 2024

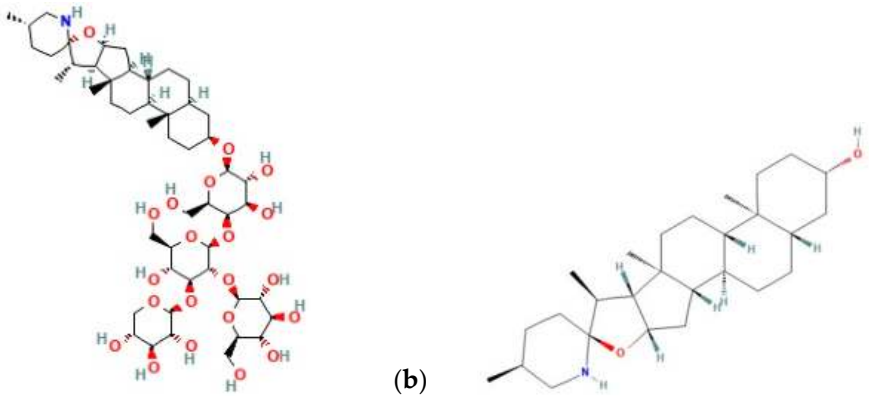
1. Introduction

Dangerous phytopathogens are usually present in the background in any agricultural setting; however, the level at which they pose a significant risk to valuable agricultural crops can vary throughout the year and is related to climactic conditions. This is an important consideration in the European part of Russia, where many factors can make a significant contribution to reducing the effectiveness of plant protection measures [1]. Indeed, many countries have a list of phytopathogenic microorganisms which are subject to strict export controls. The presence of one of these organisms, *Clavibacter michiganensis* VKM Ac-1402, is especially concerning and requires immediate quarantine of goods in both Europe and the Russian Federation [2].

Clavibacter michiganensis is a Gram-positive actinobacterium that causes diseases of many agricultural crops, the most important of which is tomato wilt. Moreover, plants affected by *Clavibacter* exhibit different symptoms depending on the age of the plant, its variety, environmental conditions, and the activity of the pathogen itself [3]. Based on the description by Wang et al. [4], in the case of seed lesions, the pathogen directly invades the vascular tissue of tomato seedlings and causes wilting of the plant. In case of infection through stomata, including hydatodes, the pathogen causes necrosis of leaf margins and

their partial wilting. According to the same source, in the early stage of infection, the plant is characterized by unilateral wilting, or shows the effect of a burnt leaf margin first on the underside and then on both sides. As the disease progresses, the entire seedling wilts. At a late stage, the affected stem splits, and ulcers with brown and hollow vascular bundles form. It should be noted that unilateral wilting of leaves and plants in the early to middle infection phase is a unique phenomenon for tomato bacterial canker, clavibacteriosis [4]. Information on the molecular genetic mechanisms of *Clavibacter* pathogenic function is of great importance for the development of plant protection products.

The following molecular mechanism of pathogenicity of *C. michiganensis* is suggested: strain NCPPPB382 contains two plasmids (*pCM1* (27 bp) and *pCM2*) that carry genes responsible for virulence [5]. In this case, there is a large region of the chromosome with a low G + C pair content, which can be divided into two regions: *chp*, a region that contains several serine proteases, and *tomA*, a cluster of genes encoding proteins that are involved in the uptake of sugars and biosynthesis of an enzyme designated as tomatinase. This enzyme may be a crucial virulence factor because tomatins are saponins that belong to a group of specialized plant metabolites contained in tomato tissues (Figure 1) and play a role in defense against pathogens [6].



(**a**) (**b**)

Figure 1. The structure of the molecules of saponin α-tomatine (**a**) and tomatidine (**b**), shown on PubChem in the public domain [https://pubchem.ncbi.nlm.nih.gov/compound/28523; https://pubchem.ncbi.nlm.nih.gov/compound/65576, accessed on 19 March 2024].

Infection and colonization of tomato fruit by *Clavibacter* can occur at all stages of growth, including flowering and fruit set. However, according to Wang Y. et al. [4], no cases of tomato flower infection have been reported. The same source provides information that fruit contamination plays a more important role than stem lesions.

During infection, *Clavibacter* encounters the defense mechanisms of the host plants. In the work of Balaji et al. [7], it has been described that infection triggers a cascade of molecular genetic processes that leads to the formation of reactive oxygen species, changes in the pH of the medium, activation of protein kinases, and induction of genes responsible for pathogenesis. The plant's response to infection by *Clavibacter* can be initiated by signaling molecules, or the so-called molecular patterns of cell components, designated as MAMPs/PAMPs and DAMPs (microbe- or pathogen-associated molecular patterns, damage-associated molecular patterns), and specialized receptor–kinases [8]. Among the signaling molecules that act as inducers of immune processes is peptidoglycan, which is an obligatory component of Gram-positive bacteria cells [9]. There is evidence that tomato sprouts treated with peptidoglycan were more resistant to plant pathogens [9]. This suggests that the plant's defense system is activated against bacterial infection when it detects the components of the bacterial cell wall, which are present in peptidoglycan. In addition, a mechanism of induced resistance has been described for plants, which, in the case of tomato, may be related to the release of jasmonic acid or ethylene [10]. Thus, plants, in particular tomato, can resist infection caused by pathogens. However, such

systems are not always effective, as phytopathogens are capable of adaptation and defeat weakened organisms.

In agriculture, plant protection products are used to prevent plant diseases. Remedies can be divided into chemical or biological solutions, but a combination of these options is often found. Preparations based on microorganisms and their secondary metabolites can exhibit antagonistic properties against pathogens but generally do not have any negative effects on the plants themselves. In some cases, the strain used even stimulates the growth of the root system or aerial parts. According to the latest data from the Russian Federation State Catalog of Pesticides and Agrochemicals, as of 22 August 2023, microorganisms are used as active ingredients in plant protection products, specifically from 11 different genera: *Beauveria, Streptomyces*, *Lecanicillium*, *Metarhizium*, *Bacillus*, *Trichoderma*, *Methylobacterium*, *Pseudomonas*, and *Acremonium*. However, in 2022, there were only three genera: *Bacillus*, *Trichoderma*, and *Pseudomonas* [11]. The range of microorganisms may increase due to new promising strains. For example, there are reports that the bacterium *Bacillus pumilus is* able to suppress phytopathogenic fungi: *Arthrobotrys conoides*, *Fusarium solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Fagopyrum esculentum*. In addition, the authors report that this microorganism is capable of stimulating plant growth [12]. Thus, it remains necessary to search for new microorganisms, study their properties, and also create collections for the development of biotechnological processes.

For instance, an isolate identified as *Janthinobacterium lividum* was obtained from the Vezelka River in Belgorod and deposited in the All-Russian Collection of Microorganisms under the reference number VKM B-3705D. Previous research revealed that this isolate exhibited mycostatic activity against two molds, *Alternaria brassiciccola* and *Aspergillus unguis*. The strain *J. lividum* VKM B-3705D was effective against *Pseudomonas putida* KT2442, *P. fluorescens* B 849, *Micrococcus luteus* B1891, and *Achromobacter ruhlandii* B-1330. Based on these findings, *Janthinobacterium* could be a promising candidate for use in plant protection products. However, it is necessary to expand the range of test cultures of phytopathogenic microorganisms to fully assess the possibility of using *J. lividum* VKM B-3705D.

The purpose of this study was to evaluate the efficacy of *J. lividum* VKM B-3705D and the pigment violacein as a biological control agent for *Clavibacter michiganensis* VKM Ac-1402 and to suggest a possible mechanistic explanation for this antimicrobial effect.

2. Materials and Methods

2.1. Bacterial Strains and Materials

Clavibacter michiganensis VKM Ac-1402 was obtained from the All-Russian Collection of Microorganisms. *Janthinobacterium lividum* strain was isolated by us earlier and was deposited in the All-Russian Collection of Microorganisms as *J. lividum* VKM B-3705D [13,14]. High-purity-grade (>98%) ethanol, isopropyl alcohol, dimethyl sulfoxide (DMSO), and acetone were obtained from Sigma-Aldrich (Burlington, MA, USA). All the other reagents were of analytical grade.

2.2. Evaluation of Sensitivity of Clavibacter michiganensis VKM Ac-1402 to Janthinobacterium lividum VKM B-3705D

To assess the sensitivity of *Clavibacter michiganensis* strain VKM Ac-1402 to *Janthinobacterium lividum* VKM B-3705D, a standardized method called the cross-streak method was employed. Additionally, a modified Kirby–Bauer assay was conducted using agar disks containing *J. lividum* BKM B-3705D on an agar nutrient medium composed of 3.0% peptone, 2.0% microbiological agar, and sterile tap water.

The effect of *J. lividum* VKM B-3705D on germination of *C. michiganensis* VKM Ac-1402 on tomato fruit discs was evaluated by co-culturing the two strains (500 µL of suspension) on fruit fragments in Petri dishes. Humidity was maintained using filter paper soaked in sterile water. As a control, pure strains without impurities were also cultivated.

2.3. Evaluation the Nature of the Interaction between Clavibacter michiganensis VKM Ac-1402 and Janthinobacterium lividum VKM B-3705D

The effects of interaction between *J. lividum* VKM B-3705D and *C. michiganensis* VKM Ac-1402 cells were assessed by the change in the number of colony-forming units (*CFU/mL*) during co-cultivation. To do this, 100 µL of the culture was seeded using the serial dilution method on an agar nutrient medium (3.0% peptone) at the initial moment (0 h), at 6, 12, and 24 h. Agar plates were incubated at 25 °C for two days, and the number of germinated colonies was counted [15]. Pure cultures cultivated separately served as a control group. It has been shown that 0.9% NaCl solution is the best medium for dilution.

Colony-forming units (*CFU/mL*) were calculated using the following formula [15]:

$$N = \frac{M_a \cdot 10^n}{V}, \quad (1)$$

where M_a—arithmetic mean number of colonies on a Petri dish; n—dilution factor; V—aliquot volume, mL. To simplify calculations, the decimal logarithm of *log(CFU/mL)* was used.

Confidence intervals (Δ), representativeness error (m), mean square deviation (σ), and relative error of measurement (ε) were calculated according to the standard formulas [16].

The significance of the difference between the average values was calculated by statistical difference method [17].

To determine the logarithmic growth phase, the specific growth parameter (R) of strains in a liquid nutrient medium (3.0% peptone) was calculated using the formula [18]:

$$R = \frac{log(CFU/mL)_1 - log(CFU/mL)_0}{log(CFU/mL)_0}, \quad (2)$$

where $log(CFU/mL)_0$ is the decimal logarithm of colony-forming units (*CFU*) in 1 mL of culture fluid at the initial time of culturing, containing microbial cells at the zero hour of incubation, and $log(CFU/mL)_1$ is at the end of incubation.

The growth rate in the logarithmic phase was calculated using the formula [18]:

$$_{max} = \frac{(log\,(CFU/mL)_1 - log\,(CFU/mL)_0)}{loge(t_1 - t_0)}, \quad (3)$$

where $log(CFU/mL)_0$ is the decimal logarithm of colony-forming units (CFU) in 1 mL of culture fluid at the initial moment of the logarithmic phase, and $log(CFU/mL)_1$ is at the end.

The cell division rate constant was calculated according to the formula [18]:

$$v = \frac{(log\,(CFU/mL)_1 - log\,(CFU/mL)_0)}{log2(t_1 - t_0)}, \quad (4)$$

where $log(CFU/mL)_0$ is the decimal logarithm of colony-forming units (CFU) in 1 mL of culture fluid at the initial moment of the logarithmic phase, and $log(CFU/mL)_1$ is at the end.

Time of generation of a colony-forming unit was calculated according to the formula [18]:

$$g = \frac{1}{v}, \quad (5)$$

where g is the generation time of a colony-forming unit, v is the fission rate constant.

The formula was used to calculate the degree of inhibition of the number of colony-forming units [19]:

$$IR = \left(\frac{log(CFU/mL)_K - log(KCFU/mL)_O}{log(CFU/mL)_K} \right) \cdot 100\%, \quad (6)$$

where $log(CFU/mL)_K$ is the decimal logarithm of colony-forming units (CFU) in 1 mL in the control and $log(CFU/mL)_O$ in the experimental.

2.4. Cultivation of Janthinobacterium lividum VKM B-3705D and Preparation of Violacein Fraction in DMSO

To investigate the effect of the violacein fraction on *C. michiganensis* VKM Ac-1402, the pigment was obtained by isopropanol extraction from lyophilically dried culture of *J. lividum* VKM B-3705D. For cultivation, 100 mL of *Jantinobacterium* from the inoculum in liquid nutrient medium (composition: 10.0 g/L peptone) was loaded into a 900 mL container of liquid nutrient medium. The strain was cultured for 3 days with aeration (at 80.0%) and agitation (400 rpm) in a bioreactor (Biocanvas LFD V1.5L, Centrion, Bucheon, Republic of Korea) at 25 °C. The culture liquid containing microbial cells was frozen at −40 °C for 18 h and lyophilized using a lyophilic dryer (AK5-4.0, Bucheon, Republic of Korea). The lyophilizate was collected and extracted with isopropyl alcohol (100 mL per 1.0 g of suspension). The mixture was stirred for one hour and filtered using vacuum (filter pore diameter—0.1 μm). The procedure to re-extract the filtrate with isopropyl alcohol was repeated until a clear precipitate was obtained. The crude extract was evaporated using a rotary evaporator and the concentrate was collected and mixed with distilled water at a 1:10 ratio and allowed to stratify for 12 h. The resulting suspension was re-filtered, and the precipitate was dissolved in 99% DMSO.

The purity of the obtained extract was evaluated by thin-layer chromatography (TLC) using plates (Sorbfil (100 × 100 mm), Krasnodar, Russia). A 1:1 mixture of acetone and ethyl acetate was used as the mobile phase. The pigment content of the extract was estimated using the Bouguer–Lambert–Bera equation using the formula:

$$C = \frac{A}{\varepsilon \times l}, \quad (7)$$

where *C*—concentration of the solution, g/L; *ε*—molar extinction coefficient; *l*—thickness of the absorbing layer, cm (1 cm); *A*—optical density of the absorbing substance [20].

The pigment in the fraction was identified by nuclear magnetic resonance (NMR) recording using a 400-MR nuclear magnetic resonance spectrometer from Agilent (Palo Alto, CA, USA, 2012), under water suppression conditions and using the WET sequence.

2.5. Evaluation of Sensitivity of Clavibacter michiganensis VKM Ac-1402 to Violacene Fraction in DMSO

The sensitivity of *C. michiganensis* VKM Ac-1402 to the violacein fraction was assessed by the disk-diffusion method. For this purpose, the pigment solution in DMSO (concentration 28.4 g/L) was diluted in the ratio of water-fraction 4:0.5 and 4:0.25. A 50 μL quantity of the resulting solution was applied to a sterile paper disc (10 mm diameter) and placed in a Petri dish on 3.0% agarized peptone in 3 repetitions. The general population of suppression radius values was 32. Aqueous DMSO solution without violacein at appropriate concentrations served as a control.

Relative error of measurement (ε), mean square deviation (σ), representativeness error of the arithmetic mean (m), and confidence intervals (Δ) were calculated by standard methods specified in Section 2.9. Reliability of the difference of the average radius of the zone of culture growth suppression was calculated using Fisher's criterion [16].

2.6. Evaluation of the Effect of Violacein Fraction in DMSO on the Growth Properties of Clavibacter michiganensis VKM Ac-1402

The effect of violacein fraction in DMSO on the growth properties of the test culture was determined when cultured in liquid nutrient medium (3.0% peptone). For this purpose, a daily culture of *C. michiganensis* VKM Ac-1402 was suspended in sterile water and passaged in 200 mL of nutrient medium (100 μL of suspension each). The concentrated pigment fraction was dissolved in DMSO and diluted in water as two variances: in a water:fraction ratio of 4:0.5 and 4:0.25 (vol/vol), respectively. The amount of concentration of both solutions were in levels of 0.05%, 0.1%, and 0.15% of the total volume (vol/vol) in nutritive medium.

Seeding of *C. michiganensis* VKM Ac-1402 without the tested solutions served as a control, and DMSO diluted in the same ratios as the extract served as a control for the extractant action. After seeding, the initial optical density was measured at λ = 600 nm. Seeds were incubated under stirring (150 rpm) for 24 h, and OD was measured every 4 h. The effect on the growth properties of the culture was evaluated by the degree of inhibition of the specific OD growth of the culture at 24 h of incubation according to the modified formula:

$$IR = \left(\frac{R_K - R_O}{R_K} \right) \cdot 100\%, \quad (8)$$

where R_k is the specific OD gain at λ = 600 nm in the control case and R_o in the experimental case and is calculated as:

$$R = \frac{OD_1 - OD_0}{OD_0}, \quad (9)$$

where OD_0 is the average optical density at the initial time of cultivation, and OD_1 is the average optical density at 24 h of incubation.

Relative error of measurement (ε), mean square deviation (σ), representativeness error of the arithmetic mean (m), and confidence intervals (Δ) were calculated by standard methods specified in Section 2.9.

2.7. Evaluation of the Effect of the Violacein Fraction in DMSO on the Activity of Crude Adenosine Triphosphatase (EC 3.6.1.3) of the Bacterium Clavibacter michiganensis VKM Ac-1402

The effect of the violacein fraction in DMSO on the activity of crude adenosine triphosphatase (ATP-ase, ATP-phosphohydrolase) EC 3.6.1.3 (IU/min) was evaluated (Table S1) by the content of inorganic phosphate in the reaction mixture at the end of incubation containing ATP as a substrate for enzyme activity [21]. For this purpose, the strain of *C. michiganensis* VKM Ac-1402 was cultured in liquid nutrient medium for 7 days. The culture (20 mL) was homogenized using ultrasound (ultrasonic homogenizer) for 2 min. At the same time, the power was 100%, the maximum allowable heating temperature was 60 °C, the duration of exposure was 15 s, and the resting time was 5 s.

The homogenizate was centrifuged at 13,000× g for 30 min. Afterwards, the supernatant was removed, and 0.1 M Tris-HCl buffer (pH = 7.0) was added. The precipitate was suspended, concentrated to 1.0 mL, and re-centrifuged to reduce the concentration of associated impurities. The supernatant was removed, and the precipitate was resuspended in 0.1 M Tris-HCl. The resulting suspension was used as an enzyme preparation. To 100 µL of enzyme preparation was added 100 µL of crude extract of violacein in DMSO (undiluted, 4:0.5, 4:0.25). Sterile distilled water and DMSO diluted according to the experimental variants were used as a control. The samples were incubated at 37 °C for 10 min.

At the end of incubation, 100 µL of the mixture of enzyme preparation and test sample was added to a reaction medium containing 750 µL of 0.1 M Tris-HCl (pH = 7.0); 100 µL of bovine serum albumin (BSA, 0.1 mg/mL); 100 µL of 0.1 M $MgCl_2$-$6H_2O$ and 50 µL of 0.01 M ATP (MP Biomedicals, LLC, Illkirch Graffenstaden, France). The reaction was incubated for 10 min and stopped by adding 100 µL of 10.0% sodium dodecyl sulfate (SDS). The released phosphate from the reaction was determined using the Kirsanov method [22]. For this purpose, 6 g of ammonium molybdenum acid was dissolved in 200 mL of distilled water under constant heating. After the solution cooled, 1.0 g of ascorbic acid was added and allowed to infuse for 30 min. The obtained solution in the volume of 1 mL was added to the same amount of the test sample, kept for 15 min and measured the optical density at a wavelength of λ = 710 nm. A mixture of molybdenum ammonium molybdic acid and ascorbic acid without phosphate served as a blank sample. The optical unit data were compared with values for the standard solution of KH_2PO_4 at concentrations of 133.3 µg/mL, 13.33 µg/mL, and 1.33 µg/mL at the same wavelength.

ATP-ase activity (IU/min) was calculated as:

$$ME/min = \frac{\left(C^1_{P'} - C^0_{P'}\right)}{t_1 - t_0}, \quad (10)$$

where ${C^0}_{P'}$ and ${C^1}_{P'}$ are the initial concentration, and at the time of reaction termination, of inorganic phosphate, respectively, μg/mL, and t is the incubation duration, min.

The degree of activity suppression was calculated using Formula (10) as:

$$IR = \left(\frac{IU/min_K - IU/min_O}{IU/min_K} \right) \cdot 100\%, \quad (11)$$

where IU/min_K is the specific enzyme activity in the control variant, and IU/min_O is the specific enzyme activity in the experimental variant.

The significance of the difference was calculated using Student's criterion of reliability and difference method [17].

2.8. Atomic Force Microscopy (AFM) Study of the Influence of Violacein Extract on Morphometric Properties of Clavibacter michiganensis VKM Ac-1402 Cells

In order to investigate how the violacein fraction affected the morphometric characteristics of the cells, 1.5 mL of a two-day liquid culture of *C. michiganensis* VKM Ac-1402 was incubated for 10 min at 37 °C with pure DMSO, violacein extract in DMSO, DMSO diluted with deionized water (0.5:4), and diluted violacein extract (4:0.5). All samples were made in duplicate. The cells were incubated under standard conditions, after which 30 μL of suspension was placed on a clean slide and kept at room temperature for five minutes. The samples were gently washed twice with deionized water to remove unattached cells and then air-dried.

The AFM measurements of the cells were performed in air and at room temperature immediately after drying the smear. AFM imaging was performed using an NTEGRA Vita atomic force microscope (configuration based on Olympus IX-71 inverted optical microscope; NT-MDT, Moscow, Russia). The scanning process was carried out in semicontact error mode with a scanning frequency of 0.8 Hz, using gold-plated probes of CSG11 series (NT-MDT, Russia) with a force constant of 1.1 N/m, a radius of curvature of 10 nm, and a nominal spring constant of 5.546 N/m. All images were processed in Nova software (version 1.0.26.1443, NT-MDT, Moscow, Russia). At least 10 cells were scanned from each sample.

The length and height of the cells were measured using Nova software tools. To calculate the surface roughness, a 0.3 μm × 0.3 μm membrane surface area was scanned for each cell. Using the roughness analysis tool in the Nova program, the values of arithmetic mean roughness were calculated.

The volume (V, μm^3) and surface area (S, μm^2) of cells were measured using Gwyddion software (version 2.49, Czech Metrology Institute, Czech Republic).

The Mann–Whitney U test was used to compare differences between groups [16].

2.9. Statistical Analysis Methods

The study used a number of standard biometric methods to statistically analyze the data obtained to assess their representativeness and the reliability of differences between groups. Calculation methods were selected as a result of assessing the sample distribution [16].

To assess general parameters, confidence intervals (Δ) were calculated using the following formula:

$$\Delta = t_{st} \ldots m, \quad (12)$$

where t_{st} is the standard value of the Student's reliability criterion, and m is the mean square error of representativeness and is calculated by the formula:

$$m = \frac{\sigma}{\sqrt{n}}, \quad (13)$$

where σ is the standard deviation and is calculated by the formula:

$$\sigma = \sqrt{\frac{\sum(V - M)^2}{n - 1}}, \quad (14)$$

where V is the obtained value, M is the arithmetic mean of the totality of the obtained values, and n is the sample size.

One of the most important parameters is the relative measurement error, which was calculated using the formula:

$$\varepsilon = \frac{m}{M} \cdot 100\%, \quad (15)$$

where M is the arithmetic mean, and m is the representativeness error of the arithmetic mean.

The error was considered insignificant if it was less than 3%. Above 3% but below 5% was satisfactory, and above 5% was high.

The significance of the difference between the average values obtained as a result of co-cultivation of strains (Section 2.3) was calculated by the method of statistical differences [17].

According to the method, the difference (d) between the studied options was found. Afterwards, the arithmetic mean of the differences between the options ($\overline{d}$) was calculated. From the data obtained, the ratio $d - \overline{d}$ was calculated. We summed it up and used it to calculate the standard deviation using the following formula:

$$S_d = \sqrt{\frac{\sum\left(d - \overline{d}\right)^2}{n(n-1)}}, \quad (16)$$

where $\Sigma(d - \overline{d})^2$—sum of squares of the difference, and n—sample.

The obtained value was used in calculating Student's reliability criterion using the following formula:

$$t \frac{(\overline{x}_2 - \overline{x}_1)}{S_d}, \quad (17)$$

where $(\overline{x}_2 - \overline{x}_1)$ is the difference in the arithmetic means of the compared groups, and Sd is the standard deviation.

The difference was considered significant if the calculated reliability criterion exceeded the table values.

Reliability of the difference of the average radius of the zone of culture growth suppression (Section 2.5) was calculated using Fisher's criterion [16]:

$$F = \frac{(M_1 - M_2)^2}{\sigma_z^2} \frac{n_1 \cdot n_2}{n_1 + n_2} \geq F_{st} \{\nu_1 = 1; \nu_2 = n_1 + n_2 - 2\}, \quad (18)$$

where M_1 and M_2 are arithmetic averages of the two samples being compared; n_1 and n_2 are the number of samples being compared; ${\sigma_z}^2$ is the variance of random diversity in a one-factor variance complex composed of the two samples under study, calculated by the following formula:

$$\sigma_z^2 = \frac{\Sigma(V_1 - M_1)^2 - \Sigma(V_2 - M_2)^2}{n_1 + n_2 - 2} \quad (19)$$

where V_1 and V_2 are the values of the radius of the growth suppression zone; n_1 and n_2 are the number of samples compared.

To calculate the significance of differences between groups using the Mann–Whitney test (U-test), the numerical values of the compared samples were arranged in ascending order in one row, and the members of the general series were numbered from 1 to $N = n_1 + n_2$ [16].

For each sample, separately, we found the sum of ranks (R_u) and determined the values that reflect the relationship between the sums of ranks of two samples.

For the first sample,

$$U_1 = R_{u1} - \frac{n_1(n_1+1)}{2} \quad (20)$$

For the second sample,

$$U_2 = R_{u2} - \frac{n_2(n_2+1)}{2} \quad (21)$$

As a *U*-test, a smaller value of U was used, which was compared with the table value of U_{st}.

3. Results and Discussion

3.1. Evaluation of Sensitivity of Clavibacter michiganensis VKM Ac-1402 to Janthinobacterium lividum VKM B-3705D

The result from qualitative methods (Figure 2) revealed that the pigment produced by *J. lividum* VKM B-3705D strain has antagonistic activity against *Clavibacter michiganensis* VKM Ac-1402.

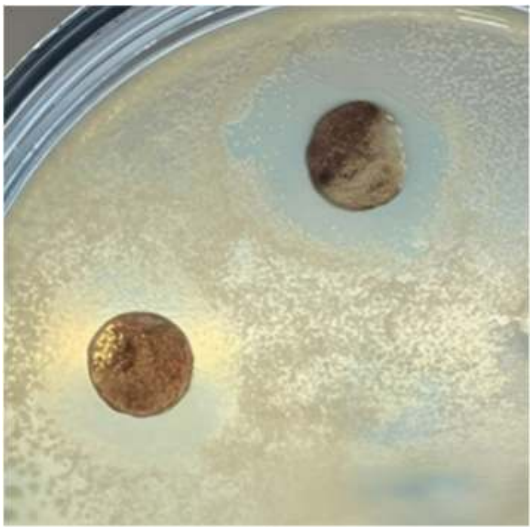
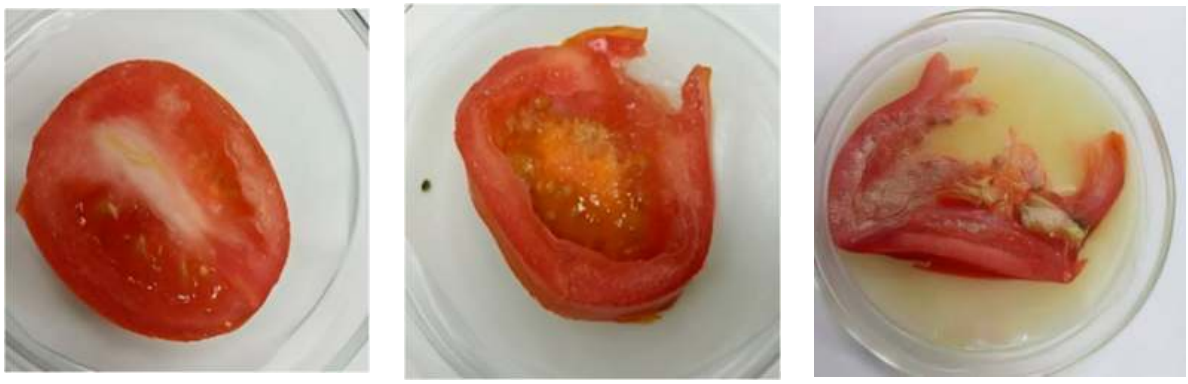


Figure 2. Sensitivity assessment of *C. michiganensis* VKM Ac-1402 to *J. lividum* VKM B-3705D by agar disc method. Each disc contained 50 µL of a cell suspension of the *J. lividum* strain grown to OD = 1.5 OU.

During the assessment of the antibacterial activity of *Jantinobacteria* using the agar disk method against *C. michiganensis* VKM Ac-1402, we observed that the test culture failed to grow in the area around the disk (Figure 2) after 24 h, with a poorly defined lawn emerging in its place, while the overgrown area did not increase in intensity over the course of a week. Thus, it can be assumed that *J. lividum* VKM B-3705D has a bacteriostatic effect against *C. michiganensis* VKM Ac-1402.

Co-cultivation of the bacteria on tomato fruit discs revealed the bacteriostatic activity of *J. lividum* VKM B-3705D against *C. michiganensis* VKM Ac-1402 (Figure 3).

Thus, it was shown by qualitative methods that *J. lividum* VKM B-3705D is able to inhibit the growth of *C. michiganensis* VKM Ac-1402 through pigment biosynthesis. During co-incubation on tomato fruit fragments, no colony development of *Jantinobacterium* colony was detected, nor was spoilage of the fragment itself (Figure 3B), whereas the inoculated tomato slice showed signs of spoilage, and *Clavibacter* significantly colonized the surface (Figure 3C).



(**A**) (**B**) (**C**)

Figure 3. Cultivation of the tested cultures on tomato, where: fruit fragment inoculated with *J. lividum* strain VKM B-3705D (**A**); fragment inoculated with a mixture of *J. lividum* VKM B-3705D-*C. michiganensis* VKM Ac-1402 (**B**); fragment inoculated with *C. michiganensis* VKM Ac-1402 (**C**).

3.2. Evaluation the Nature of the Interaction between Clavibacter michiganensis VKM Ac-1402 and Janthinobacterium lividum VKM B-3705D

During the joint cultivation of *C. michiganensis* strains VKM Ac-1402 and *J. lividum* VKM B-3705D, it was revealed that the difference in the number of bacteria (Figure 4) in the mixture at the initial moment of cultivation (0 h) was statistically significantly different by 13.0%, as calculated higher than the tabulated value at the error level of $p < 0.05$ ($t_{st} = 4.303$). At the same time, at 6 h of incubation the difference amounted to 1.5%, which turned out to be insignificant (Table S1).

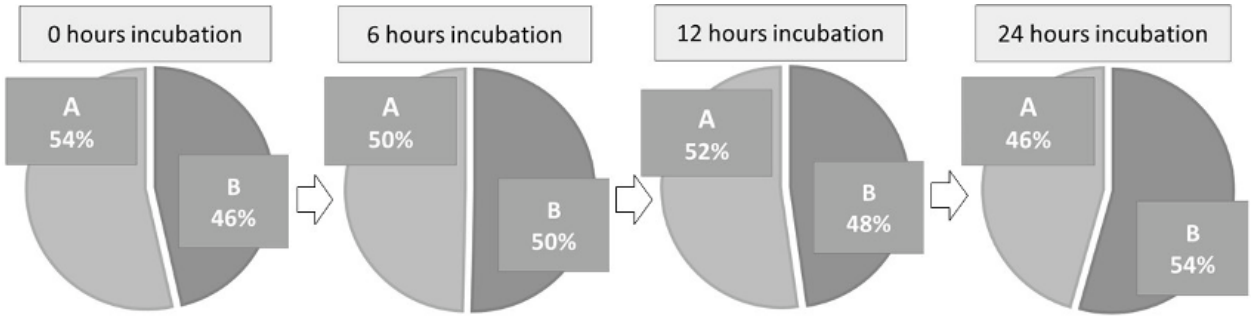


Figure 4. Colony-forming unit ratio (*log(CFU/mL)*) as an indicator of sensitivity of *C. michiganensis* VKM Ac-1402 (A) to *J. lividum* VKM B-3705D (B) when co-cultured together.

In turn, at 12 h of incubation, the abundance of *C. michiganensis* VKM Ac-1402 is statistically significantly higher than the number of *log(CFU/mL)* of *J. lividum* VKM B-3705D by 9.4%, whereas at 24 h the effect was the opposite: the abundance of *J. lividum* VKM B-3705D was higher than for *C. michiganensis* VKM Ac-1402 by 19.3% (Figure 5). At the same time, the calculated Student's t-test reliability criteria are higher than the tabulated ones at the error level of $p > 0.01$ and $p > 0.001$, respectively (Table 1).

Table 1. Colony-forming units (CFU/mL) as an indicator of growth of *C. michiganensis* VKM Ac-1402 and *J. lividum* VKM B-3705D populations in co-cultivation.

Options	Parameters				
	CFU/mL	log(CFU/mL) ± Δ	Mean Square Deviation, σ	Relative Error of Measurement, ξ%	Calculated Student's Criterion of Reliability, t
0 Hour of Incubation (Initial Values)					
Pure culture of *J. lividum* VKM B-3705D $_{(1.1)}$	1.1×10^7	7.03 ± 1.0	0.1	1.1	
Pure culture of *C. michiganensis* VKM Ac-1402$_{(1.2)}$	2.5×10^6	6.4 ± 1.1	0.1	1.4	
J. lividum VKM B-3705D culture in the presence of *C. michiganensis* VKM Ac-1402$_{(1.3)}$	3.0×10^6	6.5 ± 1.9	0.2	2.3	
Culture of *C. michiganensis* VKM Ac-1402 in the presence of *J. lividum* VKM B-3705D$_{(1.4)}$	3.5×10^7	7.5 ± 1.1	0.1	1.2	
6 h incubation					
Pure culture of *J. lividum* VKM B-3705D$_{(2.1)}$	2.85×10^7	7.4 ± 1.5	0.2	1.6	$t_{(1.1–1.3)} = 8.24$ [(a)]; $t_{(1.2–1.4)} = 6.51$ [(a)]; $t_{(1.3–1.4)} = 4.54$ [(a)]; $t_{(2.1–2.3)} = 3.87$; $t_{(2.4–2.2)} = 1.31$; $t_{(2.4–2.3)} = 0.48$; $t_{(3.1–3.3)} = 49.6$ [(c)]; $t_{(3.2–3.4)} = 5.41$ [(b)]; $t_{(3.4–3.3)} = 21.5$ [(b)]; $t_{(4.1–4.3)} = 5.2$ [(a)]; $t_{(4.2–4.4)} = 5.2$ [(a)]; $t_{(4.4–4.3)} = 38$ [(b)];
Pure culture of *C. michiganensis* VKM Ac-1402$_{(2.2)}$	1.05×10^7	7.0 ± 0.3	0.03	0.3	
J. lividum VKM B-3705D culture in the presence of *C. michiganensis* VKM Ac-1402$_{(2.3)}$	7.5×10^6	6.9 ± 0.4	0.04	0.4	
C. michiganensis VKM Ac-1402 culture in the presence of *J. lividum* VKM B-3705D$_{(2.4)}$	7.0×10^6	6.8 ± 1.6	0.2	1.9	
12 h incubation					
Pure culture of *J. lividum* VKM B-3705D $_{(3.1)}$	7.25×10^{10}	10.8 ± 1.8	0.2	1.3	
Pure culture of *C. michiganensis* VKM Ac-1402$_{(3.2)}$	2.1×10^{13}	13.3 ± 2.0	0.2	1.2	
J. lividum VKM B-3705D culture in the presence of *C. michiganensis* VKM Ac-1402$_{(3.3)}$	5.5×10^{11}	11.7 ± 1.5	0.2	1.0	
Culture of *C. michiganensis* VKM Ac-1402 in the presence of *J. lividum* VKM B-3705D$_{(3.4)}$	6.9×10^{12}	12.8 ± 0.9	0.1	0.5	
24 h incubation					
Pure culture of *J. lividum* VKM B-3705D$_{(4.1)}$	7.84×10^{11}	11.9 ± 0.4	0.05	0.3	
Pure culture of *C. michiganensis* VKM Ac-1402$_{(4.2)}$	1.5×10^{10}	10.2 ± 1.9	0.2	1.5	
J. lividum VKM B-3705D culture in the presence of *C. michiganensis* VKM Ac-1402$_{(4.3)}$	3.8×10^{13}	13.6 ± 0.4	0.04	0.2	
Culture of *C. michiganensis* VKM Ac-1402 in the presence of *J. lividum* VKM B-3705D$_{(4.4)}$	2.5×10^{11}	11.4 ± 1.1	0.1	0.8	

(a) Difference is statistically significant at $p < 0.05$; (b) difference is statistically significant at the level of $p < 0.01$; (c) difference is statistically significant at the level of $p < 0.001$.

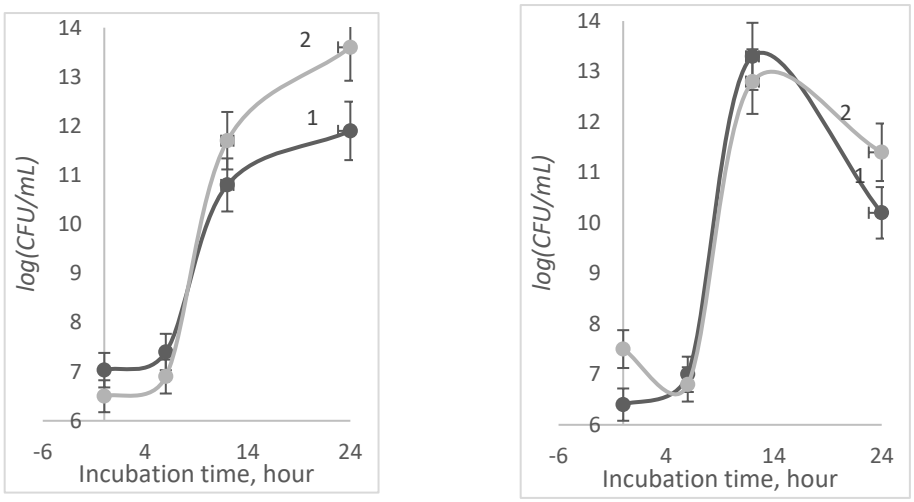


Figure 5. Change in the number of colony-forming units (*log(CFU/mL)*) *J. lividum* VKM B-3705D (**A**) and *C. michiganensis* VKM Ac-1402 (**B**) in pure (1) and mixed (2) culture during 24 h of incubation.

In a study, the effects of a mixture of different bacterial strains (*J. lividum* VKM B-3705D) on the growth of *Clavibacter michiganensis* VKM Ac-1402 were investigated. The results showed that the mixture had a significant impact on the abundance of *Clavibacter*, with an increase in the number of bacteria present. However, the number of *Clavibacter* cells decreased in the presence of the mixture, indicating that the bacteria were adapting to the mixture. This suggests that further research is needed to understand how *Clavibacter* adapts to the presence of *J. lividum* VKM B-3705D.

The comparison of growth properties of strains when co-cultured with pure strains revealed that for *C. michiganensis* VKM Ac-1402 at zero hour of incubation, the abundance in the control variant was statistically significantly lower than in the experimental variant by 17.2% ($t_{(1.2–1.4)} = 6.51 > t_{st} = 4.303$ at $p < 0.05$), and at 6 h, the value of *log(CFU/mL)* of the culture in the presence of *J. lividum* VKM B-3705D was statistically insignificantly lower than in the pure one. *J. lividum* VKM B-3705D was statistically insignificantly lower than in pure by 2.9% ($t_{(2.2–2.4)} = 1.31 < t_{st} = 4.303$). At 12 h of incubation (Table 1), the number of colony-forming units in pure culture of *C. michiganensis* VKM Ac-1402 was statistically significantly higher than for the variant in the presence of *J. lividum* VKM B-3705D by 3.8%, and the calculated Student's criterion of significance was higher than the tabulated one at $p < 0.05$ ($t_{(3.2–3.4)} = 5.41 > t_{st} = 4.303$).

In turn, at 24 h of cultivation, the difference in *log(CFU/mL)* between control and experimental variant was 11.8%, where *C. michiganensis* VKM Ac-1402 in the presence of *J. lividum* VKM B-3705D was statistically significantly higher than the abundance for *C. michiganensis* VKM Ac-1402 in pure culture, as the calculated criterion of validity exceeds the tabulated one at $p < 0.05$ ($t_{(4.4–4.2)} = 5.2 > t_{st} = 4.303$).

In the case of *J. lividum* VKM B-3705D culture, it was found that at the zero hour of incubation, the abundance in the control variant was significantly higher than in the experimental variant by 7.5% (Table 2), as the calculated Student's criterion of validity was higher than in the tabular one at $p < 0.05$ ($t_{(1.1–1.3)} = 8.24 > t_{st} = 4.303$). Moreover, at the sixth hour, the value of *log(CFU/mL)* in the control was statistically insignificantly higher than that in the experimental by 6.8% ($t_{(2.1–2.3)} = 3.87 < t_{st} = 4.303$). At 12 h of cultivation, the abundance of *J. lividum* VKM B-3705D incubated together with *C. michiganensis* VKM Ac-1402 was significantly higher than in pure culture by 7.7% ($t_{(3.1–3.3)} = 49.6 < t_{st} = 31.6$), as for 24 h, where the *log(CFU/mL)* in mixed culture exceeded the value for pure culture by 14.3% ($t_{(4.1–4.3)} = 49.6 < t_{st} = 4.303$).

Table 2. Growth kinetic parameters of *C. michiganensis* VKM Ac-1402 and *J. lividum* VKM B-3705D populations during culture.

Options	Parameters			
	Specific Grow Increase, R	**Growth Rate Constant, μ, CFU/h**	**Division Rate Constant, ν**	**Generation Time, g h^{-1}**
Pure culture of *J. lividum* VKM B-3515	0.5	0.7	1.1	0.9
Pure culture of *C. michiganensis* VKM Ac-1402	1.1	1.3	1.9	0.5
J. lividum BKM B-3515 culture in the presence of *C. michiganensis* VKM Ac-1402	0.8	1.0	1.5	0.7
C. michiganensis BKM Ac-1402 culture in the presence of *J. lividum* VKM B-3515	0.7	1.0	1.5	0.7

It has been discovered that when cultivating mixtures of bacteria, the time distribution between batches of *J. lividum* VKM B-3705D were reduced by 12 and 24 h compared to *C. michiganensis* VKM Ac-1402. This is based on the assumption that the culture is in the stationary phase of growth. All calculations of kinetic parameter were performed using data that characterize the logarithmic growth phase. Thus, the growth properties of the mixed strains deviate from the normal growth rates observed in pure strains, as shown in Figure 5.

In the course of joint cultivation, it was revealed that *C. michiganensis* VKM Ac-1402 and *J. lividum* VKM B-3705D strains were characterized by the same kinetic parameters (growth and division rate constants, as well as generation time) in the mixture, except for specific growth (Table 2). In this case, the specific growth rate of *C. michiganensis* VKM Ac-1402 in the presence of *J. lividum* VKM B-3705D was lower than that for pure culture by 36.4%. In turn, for *J. lividum* VKM B-3705D, the value of specific growth in the mixture was 37.5% higher than for pure culture.

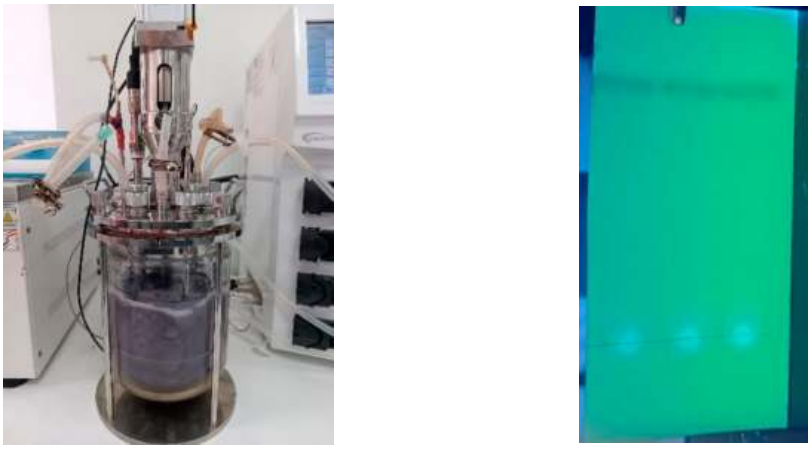
The growth and division rate constants of *C. michiganensis* strain VKM Ac-1402, relative to pure culture, decreased by 23.0% and 21.0%, respectively, during co-cultivation. The generation time increased by 28.6%.

For *J. lividum* VKM B-3705D in the presence of *C. michiganensis* VKM Ac-1402, an increase in growth and division rate constants, relative to the variant in pure culture, by 30.0% and 26.7%, respectively, was detected. The generation time decreased by 22.0%. Thus, during the joint cultivation of *J. lividum* VKM B-3705D and *C. michiganensis* VKM Ac-1402 strains, it was revealed that *Jantinobacterium* exhibited bacteriostatic activity against *Clavibacter*, as the degree of inhibition *log(CFU/mL)* was 11.8%, and the growth properties, such as growth and division rate constants, of *C. michiganensis* VKM Ac-1402 decreased relative to the control group. At the same time, the culture generation time increased.

3.3. Cultivation of Janthinobacterium lividum VKM B-3705D and Preparation of Violacein Fraction in DMSO

About 10.0 g of dried biomass was obtained by batch submerged cultivation of *J. lividum* VKM B-3705D (Figure 6A).

Extraction of the pigment with isopropyl alcohol, filtration, and re-extraction from the filtrate in DMSO, according to the Bouguer–Lambert–Bera equation, resulted in about 28.4 g/L in 99% DMSO (Table S2). The optical density of the pigment in the fraction was 1.59 ± 0.009, and the relative error of measurement was 0.21%. The purity assessment of the pigment fraction by TLC revealed that a 1:1 mixture of acetone and ethyl acetate facilitated the separation of pigment and impurities, which showed up in the UV range (Figure 6B). The 1H NMR spectrum of the sample, recorded under water suppression conditions using the WET sequence, shows resonance signals in the region of 6.7–9.0 ppm (Figure 7).



(**A**) (**B**)

Figure 6. Illustration of cultivation of *J. lividum* strain VKM B-3705D by batch submerged method in Biocanvas LFD V1.5L bioreactor, Centrion, Republic of Korea (**A**) and the result of qualitative analysis by thin-layer chromatography (TLC) (**B**).

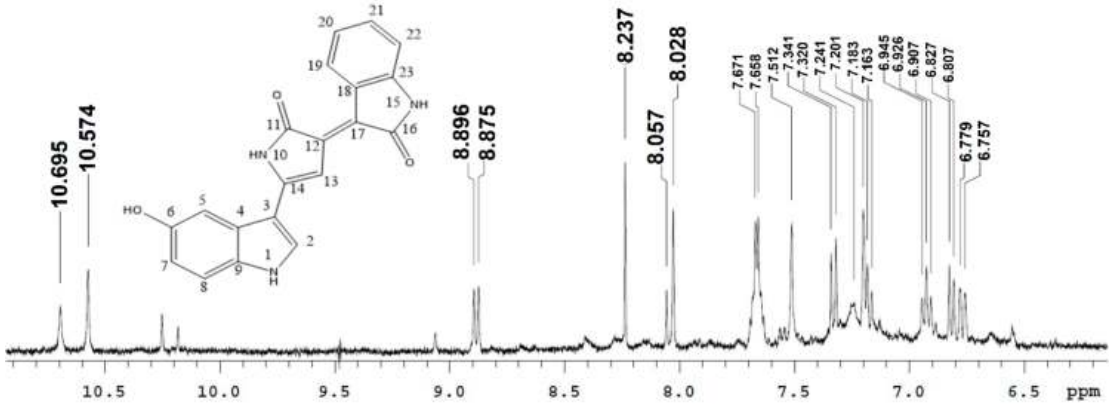


Figure 7. 1H WET 1D NMR spectrum of the aqueous extract of the *Janthinobacterium lividum* VKM B-3705D pigment.

Key signals that allow a substance to be identified as violacein [23] are indicated in the Table 3.

Table 3. Key signals that allow a substance to be identified as violacein.

Position	Signal
2	8.03 singlet
5	7.20 ppm, multiplet
7	6.77 ppm doublet, J 8.7 Hz
8	7.33 ppm doublet J 8.9 Hz
13	7.53 singlet
19	8.89 ppm doublet J 8.1 Hz
20	6.94 ppm triplet J 9.0 Hz
21	7.19 ppm multiplet
22	6.82 ppm doublet J 8.6 Hz

At present, a number of methods of violacein extraction have been developed. Various producers, both engineered and native representatives of the violacein-forming group,

have been used. It is noted that the pigment is closely associated with cells and the use of additional components—surfactants (e.g., tween-20, ionic liquids based on sodium dodecyl sulfate), glass beads, and freeze–thaw cycles—contributes to the maximum yield of violacein [24]. Information about the improvement of biosynthesis through the use of surfactant compounds is also found in other sources. For example, Gwon et al. report that when surfactant compounds (tween 80 and Triton X-100) were used, the yield of violacein was 47.64 mg/L and 48.12 mg/L, or 0.047 g/L, and 0.048 g/L, respectively [25]. The authors modeled a consortium of strains, where in one composition, there was a producer of L-tryptophan and violacein. As a result, it was possible to increase the pigment yield up to 0.083 g/L. In turn, according to the cultivation results reported by Mendes et al., 0.43 g/L was obtained, while the dry weight of the biomass was 21.0 g/L [26]. The authors used different growth substrates and their combinations. There are reports that a producer of violacein was obtained by introducing plasmid pHSX-vioABCDE into *Escherichia coli* K12DH5α, which was able to form a pigment concentration of 0.017 g/L [27]. In the paper by Duran et al., the authors reported the result of culturing a strain of *Pseudoalteromonas* sp. (DSM 13623), according to which a crude extract of pigment was obtained at a concentration of 1.1 g/L from a 30-L bioreactor [28]. The highest concentration of violacein crude extract—1.62 g/L—was obtained by the cultivation described in the work of Wang et al. [29]. B2 was cultured in medium (KNO_3—1.18 g/L, $NH_4Fe(SO_4)_2 \cdot 12 \cdot H_2O$—0.08 g/L, K_2HPO_4—0.25 g/L, $MgSO_4$—0.75 g/L, beef extract—1.53 g/L, L-tryptophan—0.74 g/L, soluble starch—13.0 g/L, pH 6.71), with a volume of 25 mL. The volume of the cultivation vessel was 250 mL, and the inoculum was 10.0%. The duration of cultivation was 32 h [29].

Thus, the study obtained a concentration of violacein in the fraction that exceeds the values reported in the literature.

3.4. Evaluation of the Sensitivity of Clavibacter michiganensis VKM Ac-1402 to the Violacein Fraction in DMSO

During the evaluation of the sensitivity of *C. michiganensis* VKM Ac-1402 to the crude extract of violacein, it was found that undiluted dimethyl sulfoxide and fractionated in DMSO had an inhibitory effect, the difference of which for these variants was statistically insignificant (Table S4), since the arithmetic mean diameter of the growth suppression zone for the pigment was 9.0% lower, and the calculated Fisher's criterion of validity was $F = 1.2$, which is lower than the tabulated values.

The violacein fraction in DMSO diluted 4:0.5 inhibited the growth of *C. michiganensis* BKM Ac-1402, while pure DMSO diluted at the same concentration had no effect on the culture. At the same time, the mean value of the radius of the inhibition zone was statistically insignificantly lower than for the concentrated variant by 10% (Fisher's calculated criterion was $F = 2.53$, which was lower than the tabulated value). The variants in the ratio of 4:0.25 were characterized by the lack of efficiency for DMSO and violacein, which was significantly lower than for the concentrated fraction (−20%). At the same time, the difference in the efficiency of the variants diluted in the ratio 4:0.5 and 4:0.25 amounted to 11%, which was statistically significant, since the calculated Fisher reliability criterion was higher than the tabular value (Table S4). The calculated concentrations of violacein in the 4:0.5 and 4:0.25 variants were 8.9 g/L and 4.7 g/L.

Thus, when the pigment fraction was diluted in DMSO, the solvent showed no inhibitory activity, and the sensitivity of *C. michiganensis* VKM Ac-1402 to the extract decreased as the violacein concentration decreased. However, the maximum averaged radius of the *Clavibacterium* suppression zone (about 3 mm) is low and does not allow us to judge the bacteriocidal activity. It is reasonable to assume that the fraction of violacein in DMSO had minimal bacteriostatic activity against continuous culture of *C. michiganensis* VKM Ac-1402.

3.5. Evaluation of the Effect of Violacein Fraction in DMSO on the Growth Properties of Clavibacter michiganensis VKM Ac-1402

In a study on *Clavibacter* cultivation in the presence of pigment in DMSO, it was observed that the variants with low concentrations did not affect the growth rate of the culture. The results showed that the readings for the control group were below 20%, and the calculated Student's reliability criteria were lower than those listed in Table 4.

Table 4. Degree of suppression of specific growth of *Clavibacter michiganensis* VKM Ac-1402 culture as an indicator of the effect of violacein fraction in DMSO at 0.05% of the medium volume.

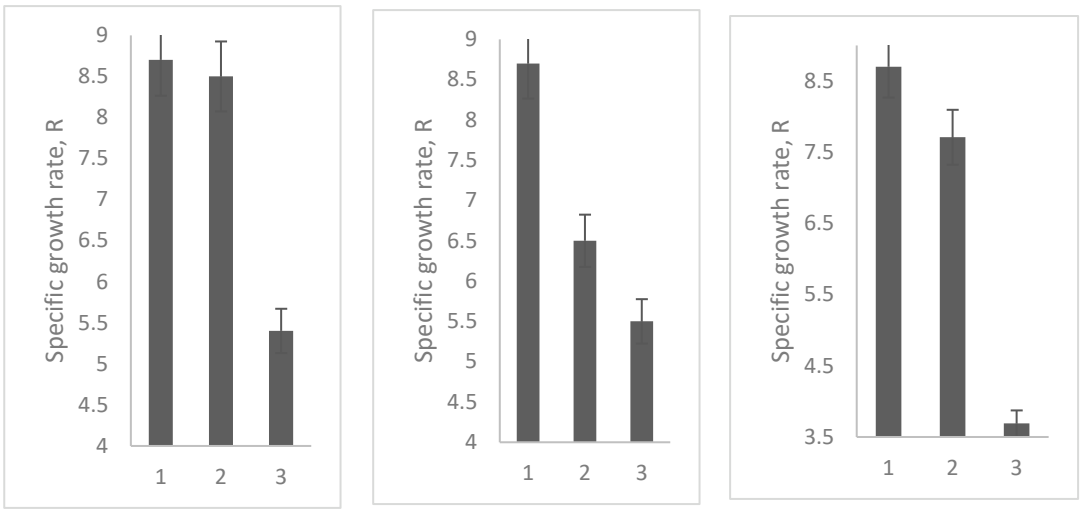
Options	Parameters				
	Arithmetic Mean at 24 h of Cultivation, $M \pm \Delta$, OD	**Relative Error of Measurement, ξ, %**	**Specific Growth Rate, *R***	**Degree of Suppression of Specific Growth, *IR*, %**	**Student's Criterion of Reliability**
Control (without test solutions)$_{(1.1)}$	0.37 ± 0.08	1.77	8.73		
DMSO undiluted$_{(1.2)}$	0.36 ± 0.04	0.81	8.47	$IR_{(1.1–1.2)}$ = 2.97%	$t_{(1.1–1.2)}$ = 1.10;
Undiluted fraction in DMSO$_{(1.3)}$	0.31 ± 0.08	2.12	5.42	$IR_{(1.1–1.3)}$ = 37.9%	$t_{(1.1–1.3)}$ = 14.70 [(a)];
DMSO–water at a ratio of 4:0.5$_{(1.4)}$	0.35 ± 0.03	0.71	7.76	$IR_{(1.1–1.4)}$ = 11.1%	$t_{(1.1–1.4)}$ = 1.67;
Fraction in DMSO–water at a ratio of 4:0.5$_{(1.5)}$	0.36 ± 0.05	1.13	7.94	$IR_{(1.1–1.5)}$ = 9.05%	$t_{(1.1–1.5)}$ = 1.00;
				$IR_{(1.1–1.6)}$ = 5.49%	$t_{(1.1–1.6)}$ = 1.57;
DMSO–water at a ratio of 4:0.25$_{(1.6)}$	0.37 ± 0.06	1.29	8.25	$IR_{(1.1–1.7)}$ = 10.42%	$t_{(1.1–1.7)}$ = 1.10;
Fraction in DMSO–water at a ratio of 4:0.25$_{(1.7)}$	0.36 ± 0.04	0.81	7.82		

(a) Difference is statistically significant at the level of $p < 0.001$.

The degree of suppression of specific growth of the test culture of *C. michiganensis* VKM Ac-1402 by undiluted DMSO in the amount of 0.05% of the medium volume amounted to 2.97%, relative to the control variant (Figure 8A). This effect was insignificant (Table 4), since the calculated Student's criterion of reliability was lower than the tabulated one at $p < 0.05$ ($t_{(1.1–1.2)} = 1.10 < t_{st} = 2.447$). In turn, the degree of suppression of specific growth of the strain in the presence, in the same amount, of the undiluted version of the violacein fraction in DMSO was 37.9% (Figure 8). At the same time, the criterion of validity (Table 4) was higher than the tabulated one ($t_{(1.1–1.3)} = 14.70 < t_{st} = 2.447$).

Evaluation of the effect of pigment on the specific growth of *Clavibacter* culture when increasing the content of the tested substance up to 0.1% (Figure 8B) of the medium volume showed that the degree of suppression of the undiluted version of the substance in DMSO amounted to 39.7%, while for the solvent itself—25.5%. At the same time, the differences of the averaged values for all samples were reliable, since the calculated Student's criteria were higher than the tabular ones (Table 4).

Increasing the content of the studied variants in the nutrient medium up to 0.15% of the total volume showed that the degree of suppression of specific growth of the undiluted pigment fraction in DMSO is significant (calculated Student's criterion of validity is higher than the tabular one, $t_{(3.1–3.3)} = 14.70 < t_{st} = 2.447$), and amounted to 57.7% (Figure 8B). In turn, for DMSO, it was 11.8 (Table 5). The criterion of validity was higher than the tabulated criterion ($t_{(3.1–3.2)} = 2.61 < t_{st} = 2.447$); hence, the difference between the solvent control and the control variant is reliable.



(**A**) (**B**) (**C**)

Figure 8. Specific growth rate of *C. michiganensis* VKM Ac-1402 in pure culture without the tested variants (1), in the presence of DMSO (2) and violacein fraction in DMSO (3), at concentrations of 0.05% (**A**), 0.1% (**B**) and 0.15% (**C**) of the medium volume.

Table 5. Degree of suppression of specific growth of *Clavibacter michiganensis* VKM Ac-1402 culture as an indicator of the effect of violacein fraction in DMSO at 0.1% of the medium volume.

Options	Parameters				
	Arithmetic Mean at 24 h of Cultivation, $M \pm \Delta$, OD	**Relative Error of Measurement, ξ, %**	**Specific Growth Rate, *R***	**Degree of Suppression of Specific Growth, *IR*, %**	**Student's Criterion of Reliability**
Control (without test solutions)$_{(2.1)}$	0.37 ± 0.08	1.77	8.73		
DMSO undiluted$_{(2.2)}$	0.3 ± 0.03	0.83	6.5		
Undiluted fraction in DMSO$_{(2.3)}$	0.28 ± 0.03	0.9	5.5	$IR_{(2.1–2.2)}$ = 25.5%;	$t_{(2.1–2.2)}$ = 8.33 [(a)];
DMSO–water at a ratio of 4:0.5$_{(2.4)}$	0.36 ± 0.05	1.13	7.89	$IR_{(2.1–2.3)}$ = 39.7%;	$t_{(2.1–2.3)}$ = 18.28 [(a)];
Fraction in DMSO–water at a ratio of 4:0.5$_{(2.5)}$	0.35 ± 0.05	1.17	8.33	$IR_{(2.1–2.4)}$ = 9.6%;	$t_{(2.1–2.4)}$ = 1.00;
DMSO–water at a ratio of 4:0.25$_{(2.6)}$	0.36 ± 0.14	3.00	8.6	$IR_{(2.1–2.5)}$ = 4.58%;	$t_{(2.1–2.5)}$ = 1.57;
Fraction in DMSO–water at a ratio of 4:0.25$_{(2.7)}$	0.36 ± 0.04	0.88	7.91	$IR_{(2.1–2.6)}$ = 1.49%;	$t_{(2.1–2.6)}$ = 0.77;
				$IR_{(2.1–2.7)}$ = 11.91%;	$t_{(2.1–2.7)}$ = 1.67;

(a) Difference is statistically significant at the level of $p < 0.001$.

The decrease in the specific growth rate may indicate that the tested samples have an inhibitory effect on population growth. Nevertheless, none of the variants exceeded its efficiency of 80%. Only the undiluted fraction of violacein in DMSO reduced specific growth relative to the control by 57.7% (Table 6). Consequently, it can be assumed that the pigment has a bacteriostatic effect on the growth of the test culture population of *C. michiganensis* VKM Ac-1402.

Table 6. Degree of suppression of specific growth of *Clavibacter michiganensis* VKM Ac-1402 culture as an indicator of the effect of violacein fraction in DMSO at 0.15% of the medium volume.

Options	Parameters				
	Arithmetic Mean at 24 h of Cultivation, $M \pm \Delta$, OD	Relative Error of Measurement, ξ, %	Specific Growth Rate, R	Degree of Suppression of Specific Growth, IR, %	Student's Criterion of Reliability
Control (without test solutions)$_{(3.1)}$	0.37 ± 0.08	1.77	8.73		
DMSO undiluted$_{(3.2)}$	0.35 ± 0.03	0.71	7.71		
Undiluted fraction in DMSO$_{(3.3)}$	0.19 ± 0.03	1.33	3.69	$IR_{(3.1–3.2)}$ = 11.8%	$t_{(3.1–3.2)}$ = 2.61 [(a)];
DMSO–water at a ratio of 4:0.5$_{(3.4)}$	0.36 ± 0.06	1.32	8.67	$IR_{(3.1–3.3)}$ = 57.7%	$t_{(3.1–3.3)}$ = 14.70 [(b)];
Fraction in DMSO–water at a ratio of 4:0.5$_{(3.5)}$	0.36 ± 0.075	1.60	8.6	$IR_{(3.1–3.4)}$ = 0.69%	$t_{(3.1–3.4)}$ = 0.24;
DMSO–water at a ratio of 4:0.25$_{(3.6)}$	0.36 ± 0.062	1.34	8.53	$IR_{(3.1–3.5)}$ = 1.49%	$t_{(3.1–3.5)}$ = 1.73;
Fraction in DMSO–water at a ratio of 4:0.25$_{(3.7)}$	0.36 ± 0.08	1.82	8.47	$IR_{(3.1–3.6)}$ = 2.29%	$t_{(3.1–3.6)}$ = 1.00;
				$IR_{(3.1–3.7)}$ = 2.99%	$t_{(3.1–3.7)}$ = 1.41

(a) Difference is statistically significant at $p < 0.05$; (b) difference is statistically significant at the level of $p < 0.001$.

Previously, it has been reported that violacein has antibiotic activity against a number of bacteria and fungi [30]. Cazoto et al. presented the results of a study showing that violacein was effective against *Staphylococcus aureus* isolates at concentrations ranging from 6.25 (0.021 mg/mL) to 25 μM (0.086 mg/mL), and in the case of test cultures of *Escherichia coli* there was no effect even at 200 μM (0.69 mg/mL) [31]. The antibacterial activity against *Staphylococcus aureus* is supported by a number of studies, among which there are variants of violacein modification to increase the solubility and quality of antibiotic property. Thus, in the work of Martins et al. the authors showed the activity of the composition of violacein and poly-(D, L-lactide-co-glycolide) against *St. aureus* and *Escherichia coli* and *Salmonella enterica* [32]. The authors confirm the fact that the pigment, even modified, is effective against Gram-positive test cultures, while Gram-negative ones did not respond to the presence of violacein. In the work of Dodou et al., the authors showed that violacein at a concentration of 20 $\mu g/mL^{-1}$ is able to inhibit the formation of biofilms and promote the degradation of the already formed film in the amount of 160 $\mu g/mL^{-1}$ [33].

Thus, the bacteriostatic activity of the violacein fraction against the test culture of *C. michiganensis* VKM Ac-1402 is consistent with examples in the scientific literature of the effect against Gram-positive microorganisms in the scientific literature.

3.6. Changes in the Morphometric Properties of Bacterial Cells in the Presence of the Violacein Fraction and Solvent

Pure solvent (DMSO) caused a large decrease in height (−42.0%, $p < 0.01$) and roughness (−56.8%, $p < 0.01$) of cells (Table S4). Diluted DMSO produced a similar but less pronounced effect (−20.7% for cell height decrease, $p < 0.05$; −27.0% for membrane roughness decrease, $p < 0.05$). When cultivated with the violacein fraction, as in the case of pure DMSO, there was a decrease in height −15.0%, $p < 0.05$) and roughness (−17.0%, $p < 0.05$), but these changes were less pronounced. The diluted violacein fraction did not cause changes in any of the measured morphometric parameters. The roughness of bacterial cell surfaces can reflect outer membrane structures and extracellular polymers [34] and often positively correlate with the degree of bacterial adhesion and biofilm formation [35]. The

joint decrease in cell roughness and height that we observed may be because the ability of cells to adhere and maintain a rigid shape is reduced, due to which the cell "sags", while maintaining the total surface area and volume.

3.7. Effect of the Violacein Fraction in DMSO on the Activity of Crude Adenosine Triphosphatase (EC 3.6.1.3) of the Bacterium Clavibacter michiganensis VKM Ac-1402

The result of the evaluation of the effect of violacein fraction in DMSO on the activity of crude adenosine triphosphatase (ATP-ase) in Table 7 revealed that the concentrated pigment significantly decreased the enzyme activity by 31.5% compared to the control group, with a calculated Student's criterion of validity that was higher than the tabulated value. Additionally, when the enzyme preparation was incubated with DMSO as a solvent control at the same concentration, the enzyme activity was significantly decreased by 8.3%.

Table 7. Activity of crude adenosine triphosphatase (ATP-ase) of *C. michiganensis* VKM Ac-1402 in the presence of violacein fraction in DMSO when the mixture was incubated for 10 min.

Options	Relative Error of Measurement, ξ, %	Enzyme Activity, IU/min	Degree of Suppression of Enzyme Activity, IR	Student's Criterion of Reliability, t
Control variant without ATP-ases (Blank)$_{(1)}$	1.68	-	$IR_{(2–3)}$ = 8.3%; $IR_{(2–4)}$ = 31.5%	$t_{(1–2)}$ = 44.8 $^{(b)}$; $t_{(3–2)}$ = 4.17 $^{(a)}$; $t_{(4–2)}$ = 11.39 $^{(b)}$; $t_{(5–2)}$ = 9.76 $^{(b)}$; $t_{(6–2)}$ = 2.1; $t_{(7–2)}$ = 1.4; $t_{(8–2)}$ = 0.87; $t_{(4–3)}$ = 16.65 $^{(b)}$
Control variant containing ATP-ase$_{(2)}$	1.63	0.1		
DMSO undiluted$_{(3)}$	1.33	0.09		
Undiluted fraction in DMSO $_{(4)}$	1.27	0.07		
DMSO–water at a ratio of 4:0.5$_{(5)}$	1.88	0.13		
Fraction in DMSO–water at a ratio of 4:0.5$_{(6)}$	1.00	0.1		
DMSO–water at a ratio of 4:0.25$_{(7)}$	0.68	0.1		
Fraction in DMSO–water at a ratio of 4:0.25$_{(8)}$	0.83	0.1		

(a) Difference is statistically significant at the $p < 0.01$ level (t_{st} = 3.169); (b) the difference is statistically significant at the $p < 0.001$ level (t_{st} = 4.587).

Nevertheless, in the presence of the fraction, a significant decrease in ATP-ase activity was observed, relative to pure DMSO by 22.0% (Table 7). Thus, it can be assumed that the violacein fraction in DMSO has a greater inhibitory effect than the solvent itself. When the concentration of violacein and the solvent itself, DMSO, was decreased, the ATP-ase activity did not change in the fractionated variant, but increased significantly by 23.0% (Table 7). Smaller concentrations of the variants had no effect on the activity of the crude enzyme.

During the study of the scientific literature, no data were found on the effect of pigments of microbial origin, including violacein, on the ATP-ase activity of *C. michiganensis*. However, the work of Cai et al. showed the inhibitory effect of the extract of *Athyrium sinense* on the ATP-ase of *Clavibacter michiganensis* subsp. *sepedonicus* [33]. It is known that ATP-ase plays an essential role in the processes of transport of substances through cell membranes, and, according to the authors, it can reflect the metabolic status of the cell. Decreased ATP-ase activity may be associated with abnormalities in the structure of the enzyme, as well as in excessive membrane permeability, which may lead to hydrolysis of ATP-ase. In addition, violacein is known to affect the cytoplasmic membranes of Gram-positive bacteria: the pigment binds to liposomes and increases membrane permeability, leading to leakage of intracellular matter such as ATP, protons and anions, resulting in osmotic imbalance [36]. Based on the available literature, it is plausible that the inhibitory

effect of the violacein fraction on the ATP-ase enzyme of *C. michiganensis* may be due to disruptions in membrane permeability. However, ATP-ase activity was measured after disruption of cells without intact membranes. It is possible that the observed effect may be attributable to changes in pH, but this assumption will be checked later.

The study revealed that the indigenous strain *J. lividum* VKM B-3705D showed bacteriostatic activity against the test culture of *C. michiganensis* VKM Ac-1402, as the co-culture of the tested microorganisms revealed that at 24 h of incubation the abundance of *C. michiganensis* VKM Ac-1402 was suppressed by the pigment-forming strain *J. lividum* VKM B-3705D. However, the same growth rate constants, division rate constants and colony-forming unit number generation time (*log(CFU/mL)*) were found for the cultures in the mixture. It can be assumed that an equilibrium in abundance was reached between *Janthinobacterium* sp. and *Clavibacter* sp. in the tank. At the same time, the specific growth of *C. michiganensis* VKM Ac-1402 in the presence of *J. lividum* VKM B-3705D was lower than for pure culture by 36.4%. For *J. lividum* VKM B-3705D, it was 37.5% higher than for pure. The growth and division rate constants of *C. michiganensis* strain VKM Ac-1402, relative to pure culture, decreased by 23.0% and 21.0%, respectively, during co-cultivation. Generation time increased by 28.6%. For *J. lividum* VKM B-3705D, an increase in growth and division rate constants in the presence of *C. michiganensis* VKM Ac-1402 was detected. It can be assumed that the bacteriostatic activity of *Jantinobacterium* sp. is the reason that together with *J. lividum* VKM B-3705D the inoculum of *C. michiganensis* VKM Ac-1402 did not give a pronounced colonization on tomato fruit fragments (Figure 3B).

J. lividum bacteria are known to produce the pigment violacein and its derivatives. Its antibiotic properties against mold fungi, bacteria, etc. are described in scientific literature. Nevertheless, we did not find data on the effect of the pigment on *C. michiganensis*, despite the fact that there are studies of the compound's effect on Gram-positive bacterial strains. Thus, during the study, a fraction of violacein in DMSO was obtained, and it was found that the highest value of the degree of suppression of specific growth of *C. michiganensis* VKM Ac-1402 population relative to the control amounted to 57.7% (a variant of undiluted fraction in DMSO, the content of which is 0.15% of the volume of the medium). Other variants showed lower efficiency. Thus, the bacteriostatic activity of *J. lividum* VKM B-3705D against *C. michiganensis* VKM Ac-1402 may be related to the ability of *J. lividum* to biosynthesize violacein.

Based on the fact that violacein showed more activity against Gram-positive test cultures than Gram-negative ones in a number of cases described in scientific papers, it can be assumed that the pigment may have an effect on the morphological properties of the cell. In evaluating such a hypothesis using scanning probe microscopy, it was found that cells aged in the violacein extract were characterized by a decrease in cell roughness and height. This may be due to the fact that the ability of the cells to retain a rigid shape is reduced, causing it to "sag" but still retain the overall surface area and volume. The pigment is known to bind to liposomes and increase membrane permeability, leading to leakage of intracellular matter [37]. This may be the cause of cell sagging.

We hypothesized that violacein has an effect on ATP-ase activity, potentially leading to increase membrane permeability and to hydrolysis of the enzyme. In the evaluation of the effect of violacein fraction in DMSO, it was found that the concentrated pigment statistically significantly decreased the enzyme activity by 31.5% relative to the control variant. At the same time, the solvent (DMSO) decreased the activity by only 8.3%. It might be assumed that the inhibitory effect of the violacein-containing fraction against crude ATP-ase of *C. michiganensis* may lead to membrane permeability disorders.

4. Conclusions

In various nations, the bacterial pathogen *C. michiganensis* is considered a significant plant pest. This work shows that *J. lividum* VKM B-3705D exhibited bacteriostatic activity against *C. michiganensis* VKM Ac-1402. It was assumed that this bacteriostatic activity is due to the pigment produced by *Jantinobacterium*. A pigment fraction identified as violacein

was obtained, which reduced the growth indexes of the phytopathogen test culture. It was found that the violacein fraction is capable of reducing the activity of the cell-free ATP-ase in *Clavibacterium*. The inhibition of ATP-ase activity may be related to changes in membrane permeability of *Clavibacterium*. However, further studies on the effect of the purified pigment, and the use of molecular genetic techniques to assess changes in gene's induction pattern are needed. In addition, the microbial cell, and its surface, is of a complex structure and contains various enzymes. It is necessary to evaluate the effect of violacein on other membrane enzymes to expand the range of possible mechanisms of bacteriostatic activity against *C. michiganensis* VKM Ac-1402.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12061116/s1, Table S1: Generalized scheme for the evaluation of the effect of violacein fraction in DMSO on the activity of crude adenosine triphosphatase (EC 3.6.1.3) of the bacterium *Clavibacter michiganensis* VKM Ac-1402.; Table S2: Absorption maximum of the violacein fraction in DMSO at wavelength λ = 575 nm as an indicator of substance concentration (g/L); Table S3: Average radius of the growth suppression zone as an indicator of the sensitivity of *C. michiganensis* VKM Ac-1402 to the violacein fraction in DMSO; Table S4: Average morphometric properties of *C. michiganensis* VKM Ac-1402 cells after the cultivation with violacein fraction and DMSO.

Author Contributions: Conceptualization, I.P.S. and N.S.L.; writing—original draft preparation, N.S.L., V.A.E., E.S.S. and A.A.A.; writing—review and editing, I.P.S. and N.S.L.; supervision, I.P.S.; project administration, N.S.L. and funding acquisition, I.P.S. All authors have read and agreed to the published version of the manuscript.

Funding: The work was carried out within the framework of the grant FZWG-2023-0007 "Adaptive responses of microorganisms: theoretical and applied aspects".

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

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