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Comparative analysis of the activity of AZ-130 and *B. subtilis* supernatants against *Lactococcus lactis*

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The aim of the presented work was to compare the activities of supernatants collected from *Bacillus vallismortis* strain AZ-130 and *B. subtilis* strains against *Lactococcus lactis* subsp. Lactis ATCC 11454. To achieve the goals, cultures of AZ-130 and *B. subtilis* and their SN were analyzed for activity against *Lactococcus lactis* subsp. Lactis ATCC 11454 by broth microdilution and growth inhibition assay. Based on the obtained results, it was found that strains AZ-130 and *B. subtilis* have high activity in the supernatant against *Lactococcus lactis* subsp. Lactis ATCC 11454 strain. However, given that the inoculation and growth conditions of both strains were the same, the activity of AZ-130 is significantly (16-fold) higher compared to the supernatant of *B. subtilis*. The presence of activity in the supernatants of AZ-130 and *B. subtilis* against *L. lactis* indicates a possible similarity of the antimicrobial compound produced by strain AZ-130 with the antimicrobial compounds produced by *B. subtilis.*

***Keywords:** Antimicrobial activity, bioactive molecules, natural products, pathogenic bacteria*

INTRODUCTION

The gradual acquisition of resistance by microorganisms to clinically used antimicrobial drugs represents a serious health problem and requires the development of new antimicrobial drugs (Armas et al., 2019; Zaman et al., 2017). Discovery and research of natural products that are produced by various organisms (plants, terrestrial vertebrates and invertebrates, marine organisms, bacteria and fungi) (Abdel-Razek et al., 2020), have provided many active and leading structures for pharmaceutical development (Schneider, 2021; Atanasov et al., 2021). Natural antimicrobials with widely varying chemical structures and biological activity play an important role in medicine, agriculture, and also in the food industry from the point of view of food safety from foodborne pathogens (Pham et al., 2019). According to Newman and Cragg 70% of antibacterial drugs on the market from 1981 to 2019 are natural products or their derivatives, 28% - synthetic drugs, 1% - imitation of natural products and pharmacophores (Schneider, 2021). Microorganisms are the most potential source for the production of natural antibacterial drugs (Wright, 2014). Isolation, purification and identification of natural antimicrobial bioactive compounds is a very time-consuming and financially demanding process (Ekins et al., 2019; Wright, 2018). However, in most cases, at later stages of development, it turns out that the molecule under study has been previously identified. One of the main steps in antimicrobial development from natural sources is to include dereplication stages in the process to avoid the re-development of already known compounds (Schneider, 2021; Carrano and Marinelli, 2015).

An AZ-130 strain, isolated from oil contaminated soil sample of Azerbaijan, showed strong activity against gram-positive opportunistic pathogenic *S. aureus* and *E. faecalis* strains

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(Araeва, 2019; Aghayeva et al., 2021) during initial and supernatant screenings. By 16S rRNA gene sequencing AZ-130 strain was identified as *Bacillus vallismortis*. Further efforts to characterize the AZ-130 bioactive compound showed that strain AZ-130 produces a single compound with antibacterial activity with a retention time at HPLC column 12.854 min (Aghayeva et al., 2021). Bacterium *B. vallismortis,* to which strain AZ-130 belongs, is very similar to *B. subtilis* (Roberts et al., 1996; Earl et al., 2012). The soil microorganism *B. subtilis* stands out among members of the genus *Bacillus* because it produces many different potential antibiotics (Caulier et al., 2019; Stein, 2005). In addition, *B. subtilis* produces a number of peptide antibiotics, including members of both classes: ribosomal synthesized (for example, subtilin, subtilosin A (Shelburne et al., 2007) ericin A and S, mersacidin, sublancin 168, bacillocin 22) (Lawton et al, 2007; Xie et al., 2009) and several types of non-ribosomally synthesized small antibiotic peptides (<2000 Da) that exhibit antibacterial and antifungal activity (for example, iturin) and lipopeptides such as surfactin, fengycin, mycosubtilin, and mycobacillin (Li et al., 2009).

The molecular weight of the AZ-130 compound is more than 3000 Da (Aghayeva et al., 2022). One of the antimicrobial compounds produced by *B. subtilis* bacterium with a molecular weight above 3000 Dalton is subtilin (3321 Da) (Subtilin). It is known, that subtilin, produced by *B. subtilis* inhibits the growth and development of *L. lactis* (Qin et al., 2019; Parisot et al., 2008). Since these two strains are closely relative to each other and may produce similar antimicrobial compounds, the purpose of the experiments presented in this work was to elucidate the similarities and differences in the mechanisms of activity of the antibacterial compounds produced by AZ-130 and *B. subtilis* strains against *Lactococcus lactis* subsp. Lactis ATCC 11454.

MATERIALS AND METHODS

The object of study was an AZ-130 antibacterial compound synthesized by the *Bacillus vallismortis* strain AZ-130 isolated from an oil-contaminated soil sample of Azerbaijan in 2014.

The *B. subtilis* strain was obtained from the Fraunhofer Mid-Atlantic Center, USA and identified by 16S rRNA gene sequencing as *Bacillus subtilis ssp. spizizenii str.* NBRC 101239.

50 ml of TB medium was added to two 125 ml flasks: one for AZ-130, the second for *B. subtilis*. Flasks were inoculated with one colony of AZ-130 (or *B. subtilis*) and incubated at 220 rpm and 32°C for 24 hours. After the incubation time, the culture was centrifuged at 10000 g for 15 min at 4° C and the supernatant was purified from the cell culture by filtration through a 0.22 μm PES membrane. Culture and supernatant of strains AZ-130 and *B. subtilis* were assayed for activity against *L. Lactis* by the growth inhibition assay. The screening was performed by the soft-agar overlay method as described by Hockett (Hockett and Baltrus, 2017; Balouiri et al., 2016) with some modifications. For screening, 10 μl of material was plated onto an agar plate confluent with the indicator strain - *Lactococcus lactis* subsp. Lactis ATCC 11454. The plates were left to dry for 5 minutes under a hood and incubated at 37°C for 24 hours. The range of antibacterial activity (zone of inhibition (ZOI)) was expressed in millimeters as the diameter of the transparent zone (the zone where the growth of the test organism was suppressed).

To compare the number of inhibitory units secreted by strains AZ-130 and *B. subtilis* over 24 hours, the collected SNs were diluted and assayed against *L. lactis* by the broth microdilution method (Manual…, 2005) according to recommendations of the Clinical and Laboratory Standards Institute (CLSI). The experiment was repeated three times. The supernatant (100 μL) was added to the first well of a 96-well plate and two-fold serially diluted across the row. Cell suspension of *Lactococcus lactis* subsp. Lactis ATCC 11454 (50 μL) was added to each well to the final concentration of 5×10^4 cells per well. For the positive control (100% growth of the test organism), 50 μL of the medium was mixed with 50 μL of the test organism suspension; pure medium without the test organism (100 μL) was used as the negative control. The plates were covered with a lid and incubated at 37°C for 22-24 hours in an open bag (to prevent moisture loss). After the incubation time, OD was measured at 650 nm using a Molecular Devices Spectra MaxPlus microplate reader.

RESULTS AND DISCUSSION

To determine the similarity/difference in the mechanism of activity of the antimicrobial compounds produced by strains AZ-130 and *B. subtilis*, SNs of AZ-130 and *B. subtilis* were analyzed for activity against *L. lactis*.

As can be seen from Figure 1, AZ-130 and *B. subtilis* show very faint activity in culture against *L. lactis*, while the SN activity of the same isolates was quite high: 8 mm – SN of strain AZ-130 and 5 mm – SN of strain *B. subtilis* (Fig. 1).

Based on the results of the growth inhibition assay, it is clear that the activity of the AZ-130 supernatant against *L. lactis* is higher compared to *B. subtilis*. It should be noted that the inoculation and growth conditions for AZ-130 and *B. subtilis* were the same. To be able to compare the number of inhibitory units secreted by these strains over 24 hours, the collected SNs were diluted and assayed against *L. lactis* by the broth microdilution method (Fig. 2).

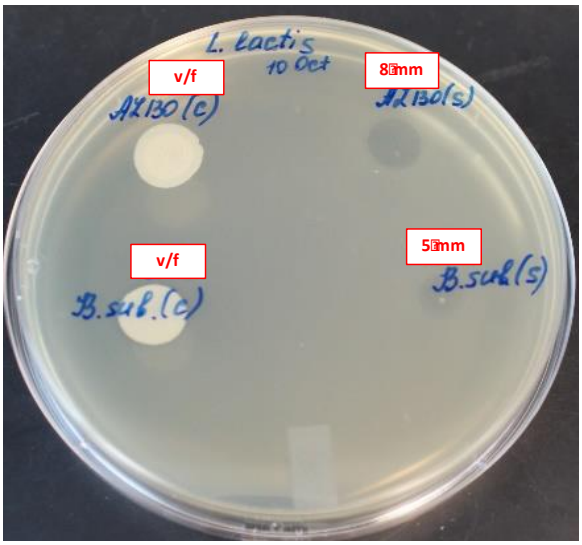

Fig. 1. Antibacterial activity of AZ-130 and *B. subtilis* cultures and their supernatants against *L. lactis*. ***Note:*** *v/f - very faint activity.*

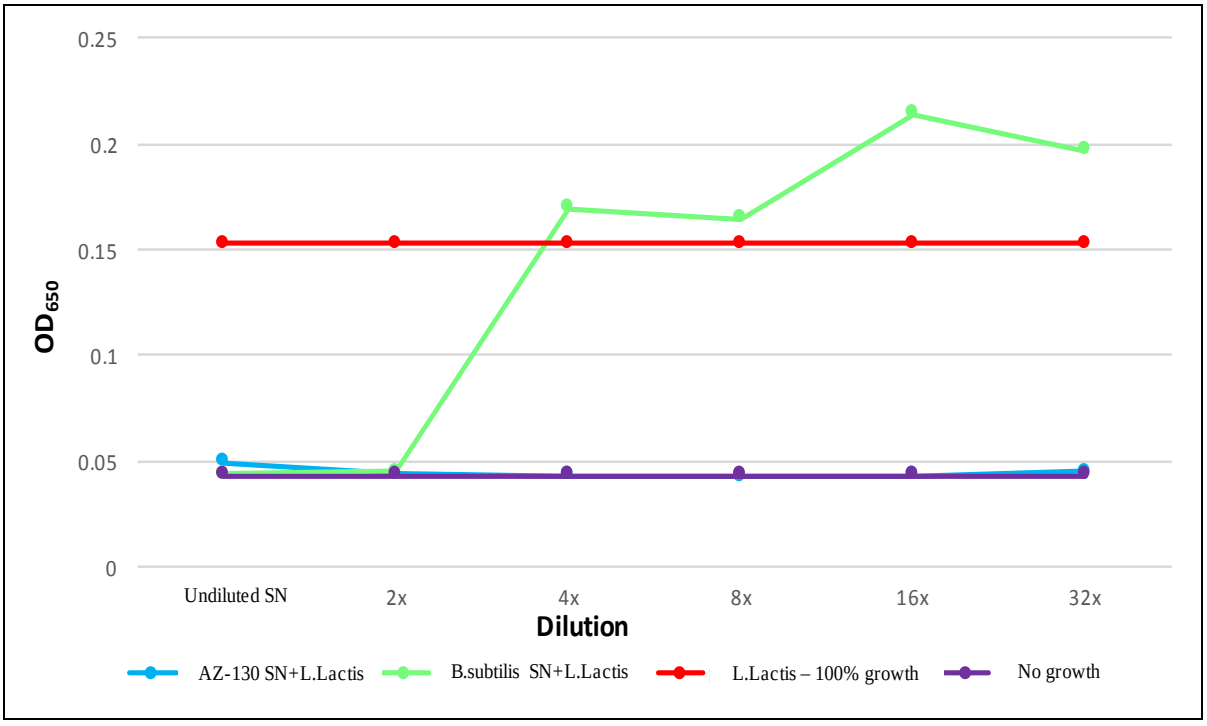

Fig. 2. Antibacterial activity of AZ-130 and *B. subtilis* cultures and their supernatants against *L. lactis*. *Note: v/f - very faint activity.*

In the figure, the maximum growth of *L. lactis* under the tested conditions (no inhibitor) is highlighted in red, the negative control (no bacterium) is in purple, the activity of the AZ-130 supernatant at various dilutions against *L. lactis* is in blue, and the activity of the *B. subtilis* supernatant at various dilutions against *L. lactis* – green. The experiment was carried out three times.

Broth microdilution analysis of the SNs (Fig. 2) showed that the AZ-130 supernatant completely inhibited the growth of the *L. lactis* strain at a 32-fold dilution, whereas the *B. subtilis* supernatant showed activity only at a 2-fold dilution. Analysis of the collected SNs by the broth microdilution method carried out to compare the number of inhibitory units, showed that the activity of the SN of strain AZ-130 against *L. lactis* was 16 times higher than the *B. subtilis* SN.

CONCLUSION

It has been found that the supernatant of strain AZ-130 has a high activity against the bacterium *Lactobacillus lactis*. The similarity in molecular mass of the AZ-130 biomolecule with subtilin produced by *Bacillus subtilis* (more than 3000 Da) and the presence of activity against *L. lactis* indicate the possibility that this antimicrobial compound belongs to the same class as subtilin, but has a 16-fold higher activity compared to subtilin.

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CONFLICT OF INTEREST

There is no conflict of interest in the present study.

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