INVESTIGATION OF ANTIMICROBIAL AGENTS PRODUCED BY GRAM-NEGATIVE BACTERIA

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Abstract
Bacterial secondary metabolites are low molecular mass compounds, which are not essential for bacterial growth. These secondary metabolites are produced in the stationary phase of bacterial growth and the produced compounds could have a variety of biological functions. One of these functions are the antibiotic or antimicrobial function that is gaining interest in the microbial community recently.
In this work Gram-negative bacterial strains were cultivated, and their produced secondary metabolites were extracted and tested in antimicrobial plate assays against Gram-positive and Gram-negative bacterial strains as well. The well-known antibiotic, pyrrolnitrin content of the extracts were also determined by HPLC-Quadrupole-Orbitrap MS.

Introduction
Bacterial secondary metabolites are low molecular mass compounds, which are not essential for bacterial growth. They provide many different biological functions for bacteria in nature [1]. There are many compounds that can be considered as secondary metabolites from bacterial sources such as antibiotics [2], enzymes inhibitors [3] and growth promoters [4]. These types of metabolites are produced during the stationary phase of bacterial growth.
Natural antibiotics are compounds that are produced by certain types of bacteria as secondary metabolites [5]. They began to take a wide range of interest especially in the medical and microbiological fields [6,7].

Experimental
Five bacterial strains (*Serratia marcescens*, SZMC 0567; *Serratia plymuthica*, SZMC 24063; *Pseudomonas chlororaphis*, SZMC 24067; *S. plymuthica*, SZMC 24069; *S. plymuthica*, SZMC 24070) were collected from the Szeged Microbial Collection (SZMC).
Strains were cultivated on three different media to get the antimicrobial effects of their secondary metabolites tested. The three media were Czapek-Dox broth (CzDb), glutamate-nitrate medium (GNM) and glutamate-nitrate medium completed with D-tryptophan (GNM+Trp).
Grown cultures were centrifuged and the supernatant was extracted with ethyl-acetate. Organic phases were dried over $MgSO_4$ and were filtered as well as evaporated.
Gained crude extracts were redissolved in 10% methanol (in water (v/v%)) and were centrifuged to get rid of the precipitate. With the solutions antimicrobial microplate assays were performed against three Gram-positive and three Gram-negative bacteria, to get their antimicrobial properties determined. After 24h of incubation, inhibitory rates were calculated.
The pyrrolnitrin content of the crude extracts was determined by HPLC-Quadrupole-Orbitrap MS. 1 mg of each crude extract was redissolved in 1 ml MS grade methanol and was injected

to the HPLC-QOMS. The pyrrolnitrin content was determined by ESTD calibration in the range of 25-500 ng/ml concentration.

Results and discussion

After successful cultivation of the five bacterial strains in three different media the fermentation material was extracted by ethyl-acetate. The antimicrobial activity of the crude extracts was tested against Gram-positive (*Bacillus subtilis*, *Micrococcus luteus* and *Saphylococcus aureus*) and Gram-negative (*Eserichia coli*, *S. marcescens* and *Pseudomonas aeruginosa*) bacteria as well. The experiments resulted that the extracts were active against Gram-positive bacteria and were no growth inhibition against Gram-negative ones. The samples were most active against *B. subtilis* and less active in the cases of *M. luteus* and *S. aureus*. There were two of the cultivated bacteria that might produce high concentrations of antimicrobial agents, because when the extract was in contact with *B. subtilis* inhibitory rates reached above 80%. These bacteria were *S. plymuthica* (SZMC 24063) grown on CzDb and GNM media and *S. plymuthica*, (SZMC 24069) grown on CzDb.

For further investigation a well-known antibiotic compound, the concentration pyrrolnitrin was determined in the crude samples by HPLC-Quadrupole-Orbitrap MS. External standard calibration was carried out in the range of 25-500 ng/ml concentration. It was found that *S. plymuthica* (SZMC 24069) grown on CzDb produced high concentrations of pyrrolnitrin (1687 ng/ml) and *S. plymuthica*, (SZMC 24070) grown on GNM+Trp (868 ng/ml). The pyrrolnitrin production of SZMC 24069 can be correlated to the antimicrobial plate assay, this high concentration of an antibiotic in a solution might cause the growth inhibition of Gram-positive bacteria.

Conclusion

Cultivation of five bacterial strains on three culture media was carried out. The secondary metabolites were successfully extracted from the liquid media. The antimicrobial activity of the crude extracts was tested against Gram-positive and Gram-negative bacteria.

It can be concluded that two bacteria had produced secondary metabolites on certain culture media that are effective against Gram-positive bacteria. The pyrrolnitrin concentrations were also determined by HPLC-Quadrupole-Orbitrap MS , and in one case, the antimicrobial activity can be correlated to the measured high concentration of pyrrolnitrin.

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