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INFLUENCE OF CULTURE CONDITIONS ON THE ANTIBIOTIC PRODUCTION OF ANTAGONISTIC BACILLUS STRAINS ISOLATED FROM TOMATO RHIZOSPHERE

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ABSTRACT - Influence of culture conditions on the antibiotic production of antagonistic *Bacillus* strains isolated from tomato rhizosphere.
Many phytopathogenic bacteria and fungi attack tomato plants both in seedling (e.g. *Rhizoctonia solani, Pythium debaryanum*) and in developed foliar state (e.g. *Phytophthora infestans*, *Botrytis cinerea*, *Alternaria solani, Xanthomonas vesicatoria*, *Pseudomonas syringae pv. tomato*, *Clavibacter michiganensis*). It would be desirable to find an efficient biocontrol approach for preventing the destructive effect of these pathogens. In the frame of this study, more than 250 bacteria with antagonistic properties were isolated and characterized. Results of the preliminary antagonism tests revealed that the strains belonging into the genus *Bacillus* were the most efficient agents against the tomato pathogenic bacteria and fungi.
The *Bacillus* strains with the best antagonistic traits were investigated in detail. The antibiotics present in the cell-free ferment broths were detected and analysed by thin layer chromatography. Specific PCR-based approach was developed and used for the detection of the distinct antibiotic-synthesis gene clusters (iturin, surfactin, fengycin, bacillomycin and mycosubtilin) in the genomes of the strains. Our investigation revealed that the best antagonistic strains produced high amount of surfactin and/or fengycin antibiotics. On the basis of these experiments two strains were selected for further investigations. The influence of the Cu^{2+} and Fe^{2+} ions and the quality of carbon and nitrogen sources were tested in liquid culture for the antibiotic production levels by the strains. Both copper and iron highly elevated the production rate at least of the tyrosine containing antibiotics. The applied carbon and nitrogen sources highly influenced both the quantity and quality of the antibiotic mixture secreted by the strain B23 of *Bacillus subtilis.*

Keywords: *Bacillus*, antibiotics, antagonism

INTRODUCTION

Many phytopathogenic bacteria and fungi attack tomato plants both in seedling (e.g. *Rhizoctonia solani, Pythium debaryanum*) and in developed foliar state (e.g. *Phytophthora infestans*, *Botrytis cinerea*, *Alternaria solani, Xanthomonas vesicatoria*, *Pseudomonas syringae pv. tomato*, *Clavibacter michiganensis*). It would be desirable to find an efficient biocontrol approach for preventing the destructive effect of these pathogens. Of the biological control alternatives to chemical pesticides used for reducing plant diseases, the application of non-pathogenic soil bacteria at roots is promising. Treatments with these beneficial organisms were in many cases associated with reduced plant diseases in greenhouse and field experiments. These bacteria can antagonize first of all fungal pathogens by competing for niche and nutriments, by producing low-molecular-weight fungitoxic compounds and extracellular lytic enzymes, and indirectly, by stimulating the defensive capacities of the host plant. Powerful antifungal metabolites can be synthesized

by most of the *Bacillus* strains. It was suggested that antibiotic production by these strains plays a major role in plant disease suppression (LECRERE at al. 2005).

MATERIAL AND METHOD

The antibiotics present in the cell-free ferment broths were detected and analyzed by thin layer chromatography (TLC). The amount of the secreted tyrosine containing antibiotics in these ferment broths after producing crude antibiotic preparates were evaluated with optical density measurement at 280 nm. For the molecular experiments DNA samples were isolated from the strains, and PCR-based approach was developed and used for the detection of the distinct antibiotic-synthesis gene clusters (iturin, surfactin, fengycin, bacillomycin and mycosubtilin) in the genomes of the strains. The PCR reactions were performed with specific primers (*Table* 1). The applied original medium for antibiotic production (BESSON et al., 1987): (constituents in g/l) glucose 10, glutamic acid 5, KH_2PO_4 1, K_2HPO_4 1, $MgSO_4$ x $7H_2O$ 0,5, KCl 1, $FeSO_4$ x 7 H_2O 0,005, $CuSO_4$ x $5H_2O$ 0,00016.

Table 1. Sequence of the primers which were used in the specific PCR reactions

Target Gene	Primer Pairs	Sequence
iturin	ituD-F	5' -ATG AAC AAT CTT GCC TTT TTA- 3'
	ituD-R	5' -TTA TTT TAA AAT CCG CAA T- 3'
surfactin	sfp-F	5' -ATG AAG ATT TAC GGA ATT TA- 3'
	sfp-R	5' -TTA TAA AAG CTC TTC GTA CG- 3'
fengycin	fen-F	5' -GTA CAG CTC GCC GAA TTC TT- 3'
	fen-R	5' -GGC TAC AAT ATG CCG GCT GTG- 3'
mycosubtilin	mycA-F	5' -GAC TGG GAT TTA TCC CAT ATC- 3'
	mycA-R	5' -GAT TTT GGT TGA CTC TAG CGC 3'
bacillomycin	BACC-ML-F	5' -CAG AGA GTC TAT CAT TCC GGA T- 3'
	BACC1-R	5' -CGC TGA TGA CTG TTC ATG CT- 3'

RESULTS

In the frame of this study, more than 250 bacteria with antagonistic properties were isolated and characterized by *in vitro* antagonism tests. Results of the antagonism tests revealed that the strains belonging into the genus *Bacillus* were the most efficient agents against the tomato pathogenic bacteria and fungi.

The *Bacillus* isolates with the best antagonistic traits were investigated in detail. Mostly the *Bacillus subtilis* strains produce a variety of antimicrobial cyclic lipopeptides, including iturin, fengycin and surfactin. The details of the above mentioned examinations were compared with the antagonistic properties of the strains on solid culture media. Our investigation revealed that the best antagonistic strains produced high amount of surfactin and/or fengycin antibiotics. On the basis of these experiments two strains were selected for further investigations. The influence of the Cu^{2+} and Fe^{2+} ions and the quality of carbon and nitrogen sources were tested in liquid culture for the antibiotic production levels by the strains. Both copper and iron highly elevated the production rate at least of the tyrosine containing antibiotics (*Table 2*. and *Table 3*). A very like behavior was experienced by

others as regards the producing of iturin and surfactin by *Bacillus* strains (MAKKAR and CAMEOTRA, 2002; LIN et al., 2007).

Table 2. Influence of the Cu^{2+} and Fe^{2+} ions on the antibioticum production

Modified Besson-media	$CuSO_4.5H_2O$ content (mg/l)	$Fe_2(SO_4)_3.6H_2O$ content (mg/l)	B05 strain OD280	B23 strain OD280
Besson 1	0,16	5	0,070	0,066
Besson 2	0,16	-	0,100	0,101
Besson 3	0,08	5	0,125	0,131
Besson 4	0,32	5	0,108	0,096
Besson 5	0,64	5	0,138	0,093
Besson 6	1,28	5	0,255	0,096
Besson 7	-	5	0,068	0,047
Besson 8	0,16	2,5	0,047	0,058
Besson 9	0,16	10	0,264	0,118
Besson 10	0,16	20	0,640	0,408
Besson 11	0,16	40	0,917	0,509

Table 3. Influence of the carbon and the nitrogen source on the antibioticum production

Carbon Source	Nitrogen Source	B05 strain OD280	B23 strain OD280
-	Na-glutamate	0,097	0,087
glucose	Na-glutamate	0,162	0,048
fructose	Na-glutamate	0,153	0,288
sucrose	Na-glutamate	0,218	0,160
maltose	Na-glutamate	0,011	0,158
starch	Na-glutamate	0,272	0,293
-	Bacto peptone	0,096	0,123
glucose	Bacto peptone	0,297	0,111
fructose	Bacto peptone	0,234	0,180
sucrose	Bacto peptone	0,230	0,279
maltose	Bacto peptone	0,142	0,119
starch	Bacto peptone	0,200	0,116

The applied carbon and nitrogen sources highly influenced both the quantity and quality of the antibiotic mixture secreted by the strain B23 of *Bacillus subtilis* (*Fig.1 and Fig. 2*).

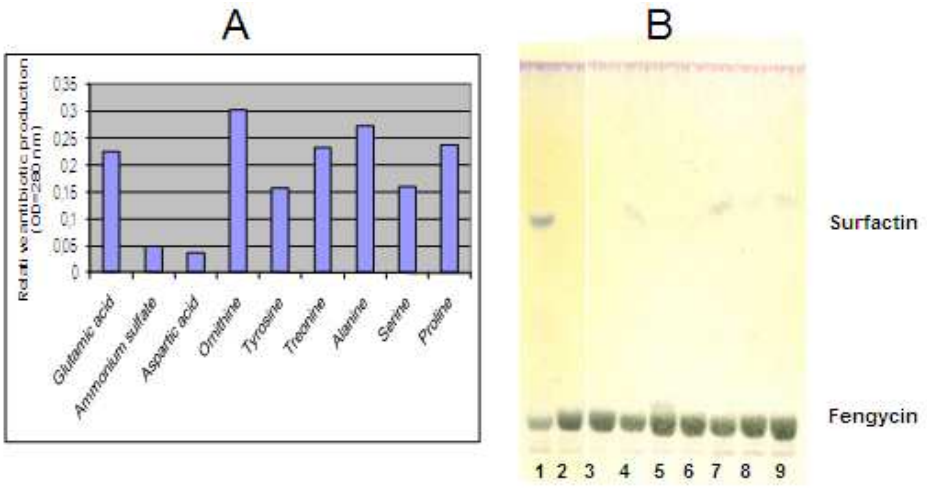

Figure 1. Influence of ammonium sulphate and some amino acids on the amount (A) and composition (B) of the secreted antibiotic mixture of *B. subtilis* B23 strain. 1=glutamic acid, 2=ammonium sulphate, 3=aspartic acid, 4=ornithine, 5=tyrosine, 6=threonine, 7=alanine, 8=serine, 9=proline. The carbon source was glucose.

Figure 2. Influence of some carbon source on the antibiotic production of *B. subtilis* B23 analysed by TLC.
G= glucose, F= fructose, S= saccharose. The nitrogen source was glutamic acid.

CONCLUSIONS

The Cu^{2+} and Fe^{2+} ions and the quality of carbon and nitrogen sources highly influenced the antibiotic production levels of the depsipeptide antibiotics of Bacillus strains. Both copper and iron highly elevated the production rate at least of the tyrosine containing antibiotics. The surfactin production rate was the high in the presence of glutamic acid as nitrogen source.

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REFERENCES

BESSON F., CHEVANET C., MICHEL G. (1987): "Influence of the culture medium on the production of iturin A by *Bacillus subtilis.*" Journal of General Microbiology **133,** 767-772**.**

LECLERE V., BECHET M., ADAM A., GUEZ J. S., WATHELET B., ONGENA M., THONART P., GANCEL F., CHOLLET-IMBERT M., JACQUES P. (2005): "Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities." Applied and Environmental Microbiology **71,** 4577-4584.

HUNG-YUH L., YERRA KOTESWARA R., WEN-SHI, W., YEW-MIN T. (2007): "Ferrous ion enhanced lipopeptide antibiotic iturin A production from *Bacillus amyloliquefaciens* B128". International Journal of Applied Science and Engineering **5,** 123-132.

MAKKAR R. S., CAMEOTRA S. S. (2002): "Effects of various nutritional supplements on biosurfactant production by a strain of *Bacillus subtilis* at 45 °C". Journal of Surfactants and Detergents **5,** 11-17.