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Influence of the culture conditions and the composition of culture medium on the antimicrobial properties of *Bacillus subtilis* strain 46/H1 against some filamentous fungi

ABSTRACT

Antibacterial and antifungal activities are characteristic features of many representatives of the genus *Bacillus*, in particular of the non-pathogenic species *Bacillus subtilis*. By using the classical agar-well diffusion method, we investigated the influence of the culture conditions and the composition of the culture medium on the antimicrobial activity of *Bacillus subtilis* strain 46/H1 against the filamentous fungi *Fusarium oxysporum* and *Aspergillus flavus*. The fungal suspensions were preliminarily inoculated in the agar media, whereas the cell-free supernatant, the cell biomass and the culture liquid of *B. subtilis* 46/H1 were pipetted into the wells. The influence of the cultural conditions on the inhibitory activity of *B. subtilis* 46/H1 was determined by cultivation in agar media with pH ranging between 5.0 and 8.0, at temperature 25°C and 30°C. The influence of the composition of culture medium on the inhibitory activity of *B. subtilis* 46/H1 was determined by changing the carbon and nitrogen sources. After 72 hours of incubation, the antimicrobial effect was determined by measuring the diameter of the zones of inhibition around the wells. The antimicrobial activity of *B. subtilis* 46/H1 against both fungal species was higher at temperature of cultivation 30oC. The change of the carbon and nitrogen sources in the standard culture medium led to an increase in the inhibitory effect, which was stronger against the fungus *Fusarium oxysporum* and almost insignificant against the second test-microorganism - *Aspergillus flavus*. In addition, the antifugal activity against *Fusarium oxysporum* was higher after changing the nitrogen source than after the change of the carbon source.

Key words: *Bacillus subtilis*, *Fusarium oxysporum*, *Aspergillus flavus*, antimicrobial activity

Introduction

The bacterial genus *Bacillus* is a large and phenotypically and genotypically heterogeneous group. Phylogenetically, bacteria from the genus *Bacillus* belong to class I of the phylum Firmicutes. Members of the genus *Bacillus* are Gram-positive, aerobic and endospore-forming bacteria that are characterized by their ubiquitous distribution, rod-shaped cell morphology and catalase production. They are found in diverse environments such as soil and clays, rocks, dust, water sources, food and the gastrointestinal tracts of various insects and animals. The ability to survive and grow in such different ecosystems is based on the production of highly resistant endospores, their physiological properties and diversity in growth requirements (Abriouel et al., 2010).

Representatives of the genus *Bacillus* exhibit quite diverse physiological properties such as the ability to degrade many different substrates derived from plant and animal sources, including cellulose, starch, proteins, agar, hydrocarbons and also biofuels. Some *Bacillus* species are heterotrophic nitrifiers, denitrifiers, nitrogen fixers, iron

RESEARCH ARTICLE

precipitators, selenium oxidizers, oxidizers and reducers of manganese, facultative chemolithotrophs, acidophiles, alkalophiles, psychrophiles, thermophiles and others. The diversity in physiological properties allowed these bacteria to colonize a wide variety of ecological habitats (Abriouel et al., 2010).

Members of the genus *Bacillus* are also considered good producers of substances with antimicrobial activity. The potential of Gram-positive endospore-forming rhizobacterium *Bacillus subtilis* to produce more than two dozen antibiotics with an amazing variety of structures has been recognized for 50 years now. The produced antimicrobial compounds include predominantly peptides that are either ribosomally synthesized and post-translationally modified (lantibiotics and lantibiotic-like peptides) or non-ribosomally generated, as well as a couple of non-peptidic compounds such as polyketides, aminosugars, and phospholipids (Stein, 2005; Lee & Kim, 2011).

In the recent few decades, the compounds produced from *Bacillus subtilis* and possessing antimicrobial activity have been extensively studied because of their potential applications in agriculture as means for control of plant diseases, in food industry as natural preservatives, in human and animal health as alternatives to conventional antibiotics, etc. (Oscariz & Pisabarro, 2000; Bizani et al., 2005).

The goal of the present study was to investigate the influence of the cultural conditions (pH and temperature) and the composition of the culture medium (by changing the carbon and nitrogen sources) on the antimicrobial activity of *Bacillus subtilis* strain 46/H1 against two important representatives of the filamentous fungi - *Fusarium oxysporum* and *Aspergillus flavus*, by the classical agar-well diffusion assay.

Materials and Methods

The following microorganisms from the collection of Department of Microbiology at University of Food Technologies, Plovdiv, Bulgaria, were used:

Microorganisms

Bacillus subtilis strain 46/H1. The strain was isolated from a natural thermal spring in Haskovo mineral spa, Haskovo district, Southern Bulgaria. The comparative 16S rRNA gene sequence-based phylogenetic analysis revealed 99% pairwise similarity of *Bacillus subtilis* strain 46/H1 to the reference strain *Bacillus subtilis* 0-2 (Tumbarski et al., 2014).

Test-microorganisms - filamentous fungi from the genera *Aspergillus* (*Aspergillus flavus*) and *Fusarium* (*Fusarium oxysporum*).

Culture media

LBG-broth medium. *B. subtilis* 46/H1 was cultured in standard LBG-medium, containing 10 g casein trypsic peptone (tryptone), 5 g yeast extract, 10 g NaCl and 10 g glucose dissolved in 1L of deionized water. The final pH was adjusted to 7.5 and medium was autoclaved for 20 min at 121°C.

LBG-agar medium. This agar medium was used for the implementation of the agar-well diffusion assay. For this purpose we prepared LBG-agar media with pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 (by the prescription above) and added 15g/L agar before autoclaving.

Modified agar media. To investigate the influence of the composition of the culture medium on the antimicrobial activity of *B. subtilis* 46/H1, we changed the carbon source (glucose) in the standard LBG-medium with fructose and the nitrogen source (tryptone) with another one of organic origin - soy peptone. They were added to the LBG-agar medium in the same quantities as above.

Malt extract agar (MEA). This medium was used for the cultivation of the test fungi. Ingredients (per 1L of deionized water): 20 g malt extract, 20 g dextrose, 6 g peptone and 15 g agar. The final pH was corrected to 5.5 and the medium was autoclaved for 10 - 15 min at 121°C.

Agar-well diffusion assay

Cultivation of test-microorganisms and preparation of spore suspensions.

The fungi were grown on MEA at 30ºC for 7 days or until sporulation. The spore suspensions were prepared by adding sterile 0.5% NaCl into the tubes and vigorous shaking. After this, the suspensions were filtered and collected. The concentration of spores in the spore suspensions was determined by using a Thoma's haemocytometer. The final concentration of spores in the suspensions for inoculation was adjusted to 1.0x105cfu/ml. Then the spore suspensions were inoculated in a preliminarily melted and tempered to 45 - 48ºC LBG-agar media. The inoculated LBG-agar media

RESEARCH ARTICLE

were transferred in quantity of 20 ml in sterilized Petri dishes (d=10 cm) and allowed to solidify. After this, six wells (d=6 mm) per dish were cut.

Cultivation of B. subtilis 46/H1 and experimental procedure.

B. subtilis 46/H1 was propagated in two tubes containing LBG-broth medium for 24 hours at 30ºC. Then the culture liquid in the first tube was stored at 4ºC and the other one was centrifuged at 3000 rpm for 10 min and the supernatant was collected. The supernatant was filtered through a bacterial filter with diameter of the pores 0.20 μm. The cell biomass was washed and resuspended in sterile 0.5% NaCl. The cell-free supernatant and the cell biomass were also stored at 4ºC. *B. subtilis* 46/H1 samples (supernatant, cell biomass and culture liquid) were pipetted in quantity of 60 μl into the agar wells in two replicates. After 72 hours of incubation at 25ºC (room temperature) and 30ºC, the antimicrobial activity was determined by measuring the diameter of the zones of inhibition around the wells.

Microorganisms with inhibition zones of 18 mm or more were considered as sensitive; moderately sensitive were those in which the zones were from 12 to 18 mm; resistant were those microorganisms where the inhibition zones were up to 12 mm or completely missing (Todorova & Kozhuharova, 2010).

Results

Influence of the culture conditions and the composition of culture medium on the antimicrobial activity of *B. subtilis* 46/H1 against *Fusarium oxysporum*.

As seen from the results in Table 1, *B. subtilis* 46/H1 had no antagonistic activity against *Fusarium oxysporum* when cultured at room temperature (25^{o}C) in standard LBG-medium with pH between 5.0 and 6.0. The antifungal effect of B. subtilis 46/H1 was moderate when it was cultured in standard LBG-medium with pH 6.5 and 7.0 at temperature 25^{o}C, and at pH from 5.5 to 8.0 at temperature 30^{o}C. The inhibitory effect was strong only when *B. subtilis* 46/H1 was cultured in standard LBG-medium with pH 7.5 and 8.0 at temperature 25^{o}C. In all cases there was no activity of the cell-free supernatant detected.

The change of the carbon source (glucose) with fructose in the composition of the standard culture medium, led to a significant increase in the inhibitory effect of *B. subtilis* 46/H1 against *Fusarium oxysporum* at both temperature conditions, except from the case when it was propagated in standard LBG-medium with pH 7.5 and 8.0 at temperature of cultivation 25^{o}C. The change of the carbon source also led to appearance of antimicrobial activity of the supernatant in the pH-range between 7.0 and 8.0.

The change of the nitrogen source (tryptone) with soy peptone in the composition of the standard culture medium, led to a significant increase in the antimicrobial activity of the culture liquid of *B. subtilis* 46/H1 against *Fusarium oxysporum* at all pH-values, especially at 30^{o}C. The change of the nitrogen source also led to appearance of moderate inhibitory activity of the supernatant in the pH-range between 7.0 and 8.0 at temperature of cultivation 30^{o}C.

Influence of the culture conditions and the composition of culture medium on the antimicrobial activity of *B. subtilis* 46/H1 against *Aspergillus flavus*.

As seen from the results presented in Table 2, *B. subtilis* 46/H1 demonstrated moderate to strong antifungal activity against *Aspergillus flavus* when cultured in standard LBG-medium, except at acidic pH of culture medium (5.0 and 5.5) and temperature 25^{o}C. Prolonged cultivation (until the 72nd hour) at 30^{o}C, led to loss of inhibitory activity of the cell-free supernatant.

The change of the carbon source in the composition of the standard LBG-medium, led to an increase in the inhibitory effect only of the supernatant of *B. subtilis* 46/H1 against *Aspergillus flavus*, at almost all pH-values, without loss of activity during prolonged cultivation (72nd hour).

The change of the nitrogen source in the composition of the standard LBG-medium, led to more sensitive increase in the antimicrobial activity against *Aspergillus flavus* at room temperature of cultivation (25^{o}C), than at 30^{o}C. The presence of soy peptone in the culture medium resulted in a retention of the antagonistic activity of the supernatant of B. *subtilis* 46/H1 up until the 72nd hour at pH-values greater than 5.5 and temperature 30^{o}C.

RESEARCH ARTICLE

Table 1. *Antimicrobial activity of B.subtilis 46/H1 against the filamentous fungus Fusarium oxysporum.*

pH	*B. subtilis* 46/H1	Inhibition zones, mm *Fusarium oxysporum* (1.0x10^5 cfu/cm^3)											
		Standard LBG-medium				Modified medium with fructose				Modified medium with soy peptone			
		25ºC		30ºC		25ºC		30ºC		25ºC		30ºC	
		48 h	*72 h*	*48 h*	*72 h*	*48 h*	*72 h*	*48 h*	*72 h*	*48 h*	*72 h*	*48 h*	*72 h*
	Supernatant	-	-	-	-	-	-	-	-	-	-	-	-
5.0	Cells	-	-	-	-	13.0	-	15.5	-	-	-	-	-
	Culture	-	-	-	-	12.5	-	13.5	-	15.5	20.0	17.5	20.0
	Supernatant	-	-	-	-	-	-	-	-	-	-	-	-
5.5	Cells	-	-	12.5	11.5	14.0	-	15.0	26.0	-	-	-	-
	Culture	-	-	10.5	10.0	14.5	-	13.5	20.5	18.5	18.5	24.0	24.0
	Supernatant	-	-	-	-	-	-	-	-	-	-	-	-
6.0	Cells	-	-	13.0	10.0	14.5	12.0	16.0	14.0	11.0	13.0	-	-
	Culture	-	-	12.5	10.0	15.0	12.0	18.5	16.0	20.0	20.0	35.5	40.0
	Supernatant	-	-	-	-	-	-	-	-	-	-	-	-
6.5	Cells	10.0	10.0	15.5	16.0	13.0	13.0	13.0	13.0	10.5	12.0	14.0	18.0
	Culture	-	-	17.0	15.5	14.5	13.0	13.0	13.0	14.0	25.0	36.0	36.0
	Supernatant	-	-	-	-	-	-	8.5	8.5	-	-	12.5	12.5
7.0	Cells	13.5	13.5	14.0	16.0	21.0	20.0	20.0	15.0	11.0	11.0	20.5	21.0
	Culture	14.0	14.0	13.5	13.5	22.0	21.0	17.5	15.5	15.0	15.0	35.0	39.0
	Supernatant	-	-	-	-	-	-	17.0	13.0	-	-	12.5	12.5
7.5	Cells	33.0	33.0	13.0	12.0	14.5	14.5	16.5	15.0	12.0	12.0	14.5	14.5
	Culture	37.0	37.0	13.5	12.0	16.0	16.0	16.0	17.5	17.0	20.0	25.0	38.5
	Supernatant	-	-	-	-	-	-	13.0	12.0	-	-	12.0	12.0
8.0	Cells	32.5	32.5	12.0	12.0	13.0	12.0	17.0	15.0	12.0	12.0	12.0	12.0
	Culture	32.5	32.5	13.0	12.0	12.0	12.0	15.0	15.0	17.0	20.0	25.0	25.0
Average value (Σ/21)		**8.21**	**8.21**	**7.62**	**7.14**	**9.97**	**6.93**	**12.3**	**10.9**	**8.26**	**8.98**	**14.1**	**15.5**

Legend: dwell = 6 mm; „-“ – no inhibition

Discussion

The results we obtained demonstrated that *B. subtilis* 46/H1 possesses highest antifungal activity against Fusarium oxysporum at 30^{o}C when fructose as a carbon source and soy peptone as a nitrogen source were used (modified LBG-media). The strongest inhibitory effect of *B. subtilis* 46/H1 against the other filamentous fungus *Aspergillus flavus* was observed at 30^{o}C when glucose and fructose as carbon sources were used, and when soy peptone as a nitrogen source was used. These results can be taken into account in the optimization of the composition of the culture media and conditions for the cultivation of *Bacillus subtilis* strains in order to favor the production of compounds with antimicrobial activity.

For the recent decades, research interest has been focused on the capacity of *Bacillus subtilis* strains to produce compounds with antimicrobial activity (bacteriocins and bacteriocin-like inhibitory substances - BLIS) and their potential applications in different fields of live.

The inhibitory effect of *Bacillus subtilis* on other microorganisms is due to the produced antimicrobial peptide, called subtilin which belongs to class I bacteriocins (lantibiotics). Besides subtilin, the *B. subtilis* strains produce other antimicrobial compounds with different structure and mode of action as the broad-spectrum bacteriocin subtilosin A, rhizocticin, surfactin, bacilysin, mycosubtilin, betacin, ericin, mersacidin, etc. (Stein, 2005; Shelburne, 2007; Abriouel et al., 2010; Lee & Kim, 2011).

RESEARCH ARTICLE

Table 2. *Antimicrobial activity of B. subtilis 46/H1 against the filamentous fungus Aspergillus flavus.*

pH	*B. subtilis* 46/H1	Inhibition zones, mm											
		Aspergillus flavus ($1.0x10^5$ cfu/cm^3)											
		Standard LBG-medium				Modified medium with fructose				Modified medium with soy peptone			
		25ºC		30ºC		25ºC		30ºC		25ºC		30ºC	
		48 h	*72 h*	*48 h*	*72 h*	*48 h*	*72 h*	*48 h*	*72 h*	*48 h*	*72 h*	*48 h*	*72 h*
	Supernatant	-	-	-	-	-	-	-	-	-	-	-	-
5.0	Cells	-	-	-	-	-	-	-	-	-	-	-	-
	Culture	-	-	25.5	22.0	15.5	15.5	15.0	14.0	-	-	-	-
	Supernatant	-	-	8.0	8.0	-	-	15.0	15.5	-	-	-	-
5.5	Cells	-	-	15.5	-	13.5	12.5	15.5	16.0	15.0	12.5	10.0	15.0
	Culture	10.0	10.0	20.5	21.0	15.0	14.5	17.0	16.5	16.5	12.0	10.0	17.0
	Supernatant	-	-	8.0	8.0	-	-	15.5	16.0	18.0	13.0	10.0	16.0
6.0	Cells	14.5	13.5	16.5	-	18.0	18.0	17.5	17.5	16.5	14.0	12.5	16.5
	Culture	15.0	14.5	30.0	30.5	18.0	17.5	18.5	18.5	16.5	15.5	13.0	16.0
	Supernatant	14.0	13.0	10.5	10.5	13.0	12.5	19.0	20.5	15.0	16.0	15.0	26.0
6.5	Cells	20.0	19.0	19.5	-	17.5	17.0	18.5	18.5	19.5	16.5	16.5	17.5
	Culture	18.0	17.5	18.5	37.0	17.0	17.5	18.5	18.5	19.0	15.5	15.0	18.5
	Supernatant	14.0	13.0	27.5	25.0	13.5	15.0	10.0	10.0	18.5	18.5	17.5	38.0
7.0	Cells	20.0	18.0	20.5	-	17.5	17.5	17.5	16.5	18.5	17.5	17.5	18.5
	Culture	19.0	19.0	31.0	27.5	18.5	17.5	18.5	17.5	18.0	18.5	18.5	18.0
	Supernatant	12.0	12.0	25.0	28.0	15.5	15.5	35.5	36.0	19.0	16.0	15.0	36.0
7.5	Cells	17.5	17.0	23.5	-	18.0	17.0	21.5	19.5	19.0	15.0	16.5	20.0
	Culture	17.0	17.0	33.5	36.5	17.5	17.0	18.0	18.0	19.5	17.5	19.0	21.5
	Supernatant	18.5	15.0	24.0	25.0	15.5	16.5	20.0	20.0	37.5	37.5	34.0	34.0
8.0	Cells	21.0	19.0	19.5	-	18.5	20.5	19.5	19.0	20.5	20.5	20.5	20.0
	Culture	19.0	17.0	33.0	37.0	18.5	17.5	18.0	19.5	20.5	20.5	21.5	21.5
Average value (Σ/21)		**11.9**	**11.2**	**19.5**	**15.0**	**13.4**	**13.3**	**16.6**	**16.5**	**15.6**	**14.1**	**13.4**	**17.6**

Legend: dwell = 6 mm; „-“ – no inhibition.

Bacillus subtilis, in particular *B. subtilis* strain 46/H1 and the antimicrobial compounds it produces, can find wide application in agriculture for biocontrol of some plant pathogens. It is well known that *Fusarium oxysporum* is among the major pathogens, which cause severe losses on most vegetables and flowers, several field crops such as cotton and tobacco, plantation crops such as banana, plantain, coffee and sugarcane, and a few shade trees (Di Pietro et al., 2003). Other fungal species as *Aspergillus flavus* are not only important phytopathogens, but also producers of extremely toxic metabolites, which can cause poisonings and even liver cancer in humans and animals when contaminated food is consumed (Moyne et al., 2001). Previous studies have also described the effectiveness of native *Bacillus subtilis* strains against *Fusarium oxysporum* (Tan et al., 2013) and *Aspergillus flavus* (Moyne et al., 2001).

The examination of the antimicrobial activity of *Bacillus subtilis* strains, is of great importance because of their possible applications as antimicrobial agents in medicine (as alternatives of conventional antibiotics) and in food industry as biopreservatives against food spoilage in different food substrates (dairy products, poultry meat, seafood products, etc.).

Using environment-friendly and food-hygienically-safe methods with plant-protecting agents of biological origin is essential for the intensive agricultural production systems. *Bacillus* strains naturally associated with soil and plants, and producing bacteriocins and/or BLIS with antibacterial or antifungal activity, could be widely applied in biological control of plant diseases, in prevention of plant decay and postharvest control of fruits and vegetables as alternatives of chemical pesticides (Foldes et al., 2000).

RESEARCH ARTICLE

Conclusion

The increasing microbial resistance to conventional antibiotics resulted in a growing interest in considering the bacteriocins synthesized by *Bacillus sp*., in particular those produced by *Bacillus subtilis*, as alternative antimicrobials against a broad spectrum of microorganisms. Bacteriocin-producing *Bacillus strains* could be widely used against different saprophytic and pathogenic microorganisms in agriculture, human and animal health and food industry. Based on the results obtained, *B. subtilis* strain 46/H1 has a potential for successful application as a bacteriocin-producing strain, and its studying have to continue in the future.

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