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STUDY OF ANTIFUNGAL ACTIVITY OF *Bacilli* SPECIES CULTURED ON AGRO-INDUSTRIAL WASTES

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Abstract. The three *Bacillus* species isolated from petroleum refinery waste were examined for antifungal activity on brewery effluents and molasses for biotechnological applications. *Bacillus* strains were identified by three different methods: 16S rRNA gene sequences, BIOLOG system and fatty acid analysis (FAME). The results demonstrated the ability of all three *Bacillus* strains cultured on brewery effluents and molasses to inhibit mycelial growth of the 10 tested fungi to varying degrees measured by agar plate inhibition assays. Fungi inhibited to the greatest degree as measured by the zones of inhibition were *Botrytis cinerea* A 258, *Phomopsis viticola* W 977, *Septoria carvi* K 2082, *Colletotrichum gloeosporioides* A 259, *Phoma complanata* A 233 and *Phoma exigua var. exigua* A 175. It was also observed that the fungal mycelial growth was inhibited by the cell-free supernatants, indicating lipoprotein-like activity of antifungal agents (mainly biosurfactants). Tested fungi were most sensitive to the *Bacilli* supernatants obtained from the molasses cultures including: *B. cinerea* A 258, *R. solani* W 70, *S. sclerotiorum* K 2291, *Phomopsis diachenii* K 657, *C. dematium* K 425, *P. complanata* A 233, *P. exigua var. exigua* A 175. In the previous study it was shown that *Bacillus* species produced biosurfactants. Application of natural products such as these *Bacillus* species or their byproducts may be a new approach to phytopathogen control therefore reducing the need for fungicides.

Key words*: Bacillus* spp.*,* Phytopathogenic fungi, Agro-industrial wastes, Biosurfactants

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INTRODUCTION

Phytopathogenic fungi affecting crop and post-harvested vegetables and fruits are a major threat to the world food supply [Spanu et al. 2010]. Typically, chemical treatments are widely used as means of control for plant pathogens [Johnson et al. 1979]. Plant growth, food quality, and crop yield are generally greater when phytopathogens are controlled. However, intensive use of the long-term toxicity and persitence chemical compounds has led to the emergence of pathogen resistance as well as detrimental environmental and human health impacts. In addition there are a number of plant diseases for which chemical agents are ineffective. In recent years biological control of plant disease, e.g. using insects and microorganisms has proven safe, cost effective, and efficient [Król 2004]; the specific application of natural antagonistic microorganisms is a promising and powerful alternative to synthetic pesticides for more rational and safe crop management [Arguelles-Arias et al. 2009, Gordillo et al. 2009]. Biological control through the use of natural microorganisms has demonstrated that yeast, fungi and bacteria can be effective against various plant pathogens [Król 2004, Perez-Garcia et al. 2011].

Gram-positive bacteria, including Bacillus *spp*., produce a variety of antibacterial and antifungal metabolites, antibiotics such as zwittermicin-A and kanosamine and cyclic lipopeptides (LPs) from the surfactin, iturin and fengycin families [Emmert and Handelsman 1999; Jacobsen et al. 2004; Ongena and Jacques 2007]. Cyclic lipopeptides have well potential in biotechnological applications because of their biosurfactant properties. Biosurfactants have numerous beneficial qualities including non-toxic, non-hazardous, biodegradable, environmental friendly, selective, effective under extreme conditions, numerous industrial applications, and unique surface-active properties. In spite of these beneficial properties, their higher production cost compared to synthetic surfactants is a major drawback. Biosurfactants could potentially replace synthetic surfactants if cost of their production was lowered substantially. The best way to reduce substrate cost for biotechnology at present is to use recycled agricultural material with the right balance of nutrients to support microbial growth and biosurfactants production for environmental applications. So far, several renewable substrates including various agricultural and industrial by-products and waste materials have been intensively studied for microorganism cultivation and biosurfactant production on the laboratory scale. These include olive oil mill effluent, waste frying oil, oil refinery wastes, soapstock, molasses, whey, starch wastes, cassava-flour processing effluent and distillery waste [Makkar and Cameotra 2002, Deleu and Paquot 2004]. Value added products or benefits can improve the economic of such bioprocesses including microbial waste reduction.

Strains of *B. subtilis* have been studied as biocontrol agents of plant pathogens growing in microbiological media [Toure et al. 2004, Ongena et al. 2005, Ongena et al. 2007, Joshi et al. 2008, Grover et al. 2010]. *B. amyloliquefaciens* which is closely related to *B. subtilis* was shown to be used as biocontrol product growing in traditional microbiological media [Mari et al. 1996; Yoshida et al. 2001, Yu et al. 2002]. Biocontrol activity of *Bacillus* strains is associated with their biosurfactants production and the ease of growth. Yu et al. (2002) isolated and identified three isomers of iturin A that inhibited *Rhizoctonia solani in vitro*. *Bacillus*-based products have great potential for

use in plant disease biocontrol. However, limited research has been undertaken to obtain information on their application in biocontrol.

The aim of the study was to evaluate antifungal activity of *Bacillus* strains growing on agro-industrial wastes and their cell-free supernatants as source of antifungal agents like biosurfactants.

MATERIALS AND METHODS

Microbial strains. The bacterial strains (T-1, T'-1 and I'-1a) used in this study were isolated from 100-year-old oil refinery sludge in Czechowice-Dziedzice (Poland) as described by Berry et al. (2006). Bacterial isolates were identified by: (1) 16S rRNA gene sequence analysis, (2) fatty acids analysis (FAME) and (3) BIOLOG™ system.

A direct-colony, PCR (Polymerase Chain Reaction) was set up to amplify the 16S rRNA gene in a 30-cycle PCR using universal primers 27F and 1492R. The PCR conditions used were: initial denaturation at 95ºC for 8 min, 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min and elongation at 72°C for 1 min, followed by elongation at 72ºC for 10 min. The amplified PCR products were purified using the Qiagen-PCR purification kit as per the manufacturer's instructions. The purified PCR products were sequenced from both ends at the DNA Sequencing Core facility of the University of Michigan, Ann Arbor. The 16S rRNA gene sequences were analysed at the Ribosomal Database Project (RDP) II (http//:rdp.cme.msu.edu). The top 10 most homologous sequences were aligned using the CLUSTALW program v1.83 at the European Bioinformatics site (www.ebi.ac.uk/clustalw). The similarity matrix was prepared using the DNAdist program in the PHYLIP package with Jukes-Cantor corrections. Isolates were identified as the genus/species to which they showed highest 16S rRNA gene sequence similarity in the RDP database.

Bacteria were identified based on whole-cell cellular fatty acids, derivatized to methyl esters (FAME) and analyzed by gas chromatography (GC) using the MIDI microbial identification system (Newark, USA). MIDI-FAME were extracted from bacteria according to the standard and recommended procedure, consisting of saponification, derivatization, extraction, and final base washing (Microbial ID Inc., 1999). FAMEs were separated by Hewlett Packard 6890 GC on a capillary column HP-Ultra 2 (cross-linked 5% diphenyl, 95% dimethyl polysiloxane; 25 m, 0.22 mm ID; film thickness 0.33 μm) with hydrogen as a carrier gas and analyzed by Sherlock 6.1 MIDI software, using the aerobe TSBA6 method and TSBA6 library (MIDI Inc., Newark, DE, USA).

The commercially available BIOLOG™ identification system was used, according to the manufacturer's specifications, to characterize utilization of various carbon substrates. The Omni log BIOLOG™ system was used with the GEN III MicroPlate™ test panel that provides 94 biochemical tests to profile and identify microorganisms. Fresh overnight cultures of the three isolates were tested as recommended by the manufacturer.

The fungal plant pathogens originated from the collection of the Department of Phytopathology and Mycology, University of Life Sciences in Lublin. They were previously isolated from various parts of caraway (*Sclerotinia sclerotiorum* K2291, *Phomop-*

sis diachenii K 657, *Septoria carvi* K 2082, *Colletotrichum dematium* K 425), angelica (*Botrytis cinerea* A 258, *Colletotrichum gloeosporioides* A 259, *Phoma complanata* A 233, P*homa exigua var. exigua* A 175) and grapevines (*Phomopsis viticola* W 977, *Rhizoctonia solani* W 70). Their taxonomic position was based on macro- and microscopic features and pathogenicity tests [Machowicz-Stefaniak et. al. 2011]. Majority of these pathogens can infect a lot of plant species but some of them such as *Phomopsis viticola*, *Septoris carvi* and *Phomopsis diachenii* infect only one host or a few species within the same botanical family. Moreover, the studied strains represented both soil pathogens and above-ground plant pathogens.

Table 1. Selected parameters of brewery effluents (A) and molasses (B) utilised in this work
Tabela 1. Wybrane parametry ścieków browarnianych (A) i melasy (B) wykorzystanych w pracy

A.

Parameters Parametr	Unit Jednostka	Brewery effluent # 4 Ściek browarniany # 4	Brewery effluent #6 Ściek browarniany # 6
pH		9.55	7.43
Conductivity	µS/cm	2000	-
COD	mgO_2/L	4280	4758
BOD_5	mgO_2/L	2850	3435
Organic matter	mg/L	467	584
SO_4^{2-}	mg/L	73.2	-
S^{2-}	mg/L	0.054	-
PO_4^{3-}	mg P/L	13.6	-
P organic	mg P/L	26.3	14.10
NH_4^+	mg N/L	8.89	-
NO_2^-	mg N/L	13.79	-
NO_3^-	mg N/L	1.03	-
Total N	mg N/L	76.5	57.00
Ca	mg/L	44.3	-
Cl^-	mg/L	106.4	-
TOC	mg C/L	1540	1869

B.

Parameters Parametr	Unit Jednostka	Molasses Melasa
pH		7.65
COD	mgO_2/L	49 075
BOD_5	mgO_2/L	25 060
Water content	%	17.75
Organic matter	%	82.15
Sucrose	%	51.25
Aminoacids	%	5.5
Total N	%	1.94

Culture conditions for antifungal tests. Bacterial suspensions of the three *Bacilli,* obtained from a nutrient agar slant incubated for 24 h at 30°C, in the liquid Standard Methods medium of the following composition ($g \cdot cm^{-3}$): peptone – 8, yeast extract – 2.5, glucose – 1 were adjusted to OD_{600nm} 0.65 (ca. 10^7–10^8 $CFU \cdot dm^{-3}$). Then, three ml of the bacterial suspensions were inoculated in 300 ml Erlenmeyer flasks containing 100 ml of the sterilized brewery effluents or 10% (v/v) of molasses as nutrients sources. The characteristics of the brewery effluents and molasses are presented in Table 1A and B. The cultures were grown aerobically at 30 ºC for 96 h with constant shaking (110 rpm). After incubation, the cultures were centrifuged at 10 000 g for 20 min. Then, the supernatants were sterile-filtered by 0.22 µm syringe filters (Sartorius, Germany) to obtain cell-free supernatants.

***In vitro* antagonism experiment.** *Bacillus* strains growing on the two brewery effluents and molasses and their culture supernatants were tested for the ability to inhibit the growth of fungal plant pathogens in Petri dishes on PDA medium as described by Velmurugan et al. [2009] with some modifications. Fresh mycelium plugs of the fungi were inoculated in the plate center, 2.5 cm from the 20 µl of the bacterial cultures which were added drop by drop on the sterilized discs (Oxoid). Then, 200 µl of the cell-free supernatants were dispensed in wells made with a sterile cork borer at 1 cm diameter made in gelified medium. Fungal growth inhibition was evaluated after plate incubation at 25°C for 3 or 10 days, depending on the pathogens growth. Sterilized media were used as the controls. Antifungal index (mycellial growth inhibition) was calculated as the following:

$$antifungal\ index\ (\%) = \left(1 - \frac{A}{B}\right) * 100\%$$

where: A – diameter of fungal growth in the treatment samples;
B – diameter of fungal growth in the controls

RESULTS AND DISCUSSION

The *Bacillus* strains and their culture supernatants were applied to test the *in vitro* antifungal effect. The morphological and biochemical characteristics of the three isolates were presented by Płaza et al. [2010]. Our early investigations confirmed that three *Bacillus* strains (T-1, T'-1 and I'-1a) were able to grow and produce biosurfactants in brewery effluents and molasses media at 30°C under aerobic condition [Płaza et al. 2010, 2011]. The identification of *Bacillus* strains by using three different methods: biochemical (i.e. FAME), metabolic (Biolog system) and 16S rDNA gene sequencing is presented in Table 2. The 16S rRNA gene sequences showed that strain I'-1a was identified as *B. subtilis*, but T-1 and T'-1 were identified as *Bacillus spp*. The 16S rRNA gene sequencing could not clearly assign isolates T-1 and T'-1 to any species of *Bacillus* as both isolates showed > 99% similarity to two distinct species (*B. subtilis* and *B. licheniformis* for T-1 and *B. subtilis* and *B. amyloliquefaciens* for T'-1). The metabolic profile of 94 biochemical tests as measured by the BIOLOG™ system,

showed identification matches for all three *Bacillus spp.* T-1 strain was assigned to the species *B. subtilis* or *B. atrophaeus* with SIM value of 0.593 and 0.549, respectively, based on it's utilization pattern of 37 substrates. The two strains T'-1 and I'-1a were identified as *B. subtilis ss spizizenii* with SIM value of 0.567 utilizing 68 substrates while *B. licheniformis* with SIM value of 0.562 utilized 50 substrates, respectively. FAME analysis showed that two strains T-1 and I'-1a were *B. subtilis*, but T'-1 was *B. amyloliquefaciens*.

Table 2. Identification of *Bacillus* strains
Tabela 2. Identyfikacja szczepów *Bacillus*

Bacillus species Szczepy *Bacillus*	Bacteria Identification Methods – Metody identyfikacji bakterii		
	biolog system system biolog	16S rRNA	FAME
T-1	*B. subtilis*/*atrophaeus*	*B. subtilis* & *B. licheniformis*	*B. subtilis*
T'-1	*B. subtilis ss spizizenii*	*B. subtilis* & *B. amyloliquefaciens*	*B. amyloliquefaciens*
I'-1a	*B. licheniformis*	*B. subtilis*	*B. subtilis*

The Bacillus strains tested in this study were characterized based on a polyphasic taxonomic approach that included phenotypic and genotypic characterization that has been demonstrated successful by other investigations [La Duc et al. 2004]. However, the taxonomic study of environmental *Bacillus* isolates has been reported to show some limited variations [Paolis and Lippi 2008]. Several authors described that 16S rDNA gene sequence analysis gave some misidentification of *Bacillus* and related strains [Guinebretiere et al. 2001, Cho et al. 2004, Wu et al. 2006]. In spite of this, the utilization of different metabolic and molecular methods is recommended to support an unambiguous identification of *Bacillus* strains isolated from different environments [Ash et al. 1993, Chun and Bae 2000, Guinebretiere et al. 2001, Suiko et al. 2004, Hutsebaut et al. 2006, Wu et al. 2006, Paolis and Lippi 2008]. As shown, the 16S rDNA gene sequence of genus *Bacillus* is phylogenetically very heterogeneous and their identification on the basis of 16S rDNA sequence analysis has been questioned. Chun and Bae [2000] demonstrated that the use of partial *gyrA* sequence, coding for DNA gyrase subunit A was a good approach in the determination of phylogenetic relationships and allowed accurant classification of *B. subtilis* and related taxa, including *B. licheniformis*, *B. mojavensis*, *B. amyloliquefaciens* and *B. atrophaeus*. The phylogenetic tree based on the neighbour-joining analysis of the *gyrA* NT sequences is presented by Chun and Bae [2000]. Authors suggested that the method for the amplification and sequencing of partial *gyrA* genes may be useful for rapid identification of *B. subtilis*-like isolates. Hutsebaut et al. [2006] used Raman spectroscopy to identify closely related species like *Bacillus* strains which belong to the phylogenetically homogeneous "*Bacillus subtilis*"-group. The evaluation resulted in 49/54 correct identification of the species.

Results presented in this paper confirm that bacilli are difficult organisms to be identified by one method and it is necessary to perform further identification tests to ascertain their taxonomic position. The results of 16S rRNA analysis seemed to confirm the close similarity T-1 and T'-1 strains to two *Bacillus* species (*B. subtilis* and *B. licheniformis* for T-1 and *B. subtilis* and *B. amyloliquefaciens* for T'-1). These results vary from the results obtained by the Biolog system and FAME. Probably, DNA-DNA hybridization could be a more accurate method for the delineation of bacterial strains like *Bacillus* isolates.

In Table 3 and Fig. 1 (A-C) mycelium growth inhibition by active *Bacillus* broth cultures is evident. The results demonstrated the ability of the *Bacillus* strains growing on brewery effluents and molasses to inhibit the mycelial growth of all tested phytopathogenic fungi. The great mycelial inhibition was observed for the following fungi: *B cinerea* A 258, *Phomopsis viticola* W 977, *Septoria carvi* K 2082, *Colletotrichum gloeosporioides* A 259, *Phoma complanata* A 233 and *Phoma exigua var. exigua* A 175.

Similar results have been observed concerning inhibition of different pathogens by *Bacillus* strains both *in vitro* and *in vivo* [Yu et al. 2002, Gordillo et al. 2009, Zhao et al. 2010]. *Bacillus spp*., particularly *B. subtilis,* are known to produce antimicrobial agents subtilin, bacilysin, mycobacillin and lipopeptides belonging to surfactin, iturin, lichenycin and fengycin families [Emmert and Handelsman 1999, Ongena and Jacques 2007]. Most of these compounds have antifungal activity, most typically attributed to iturin. This group consists of cyclic peptides such as iturin A-E, bacillomycin D, F, L, mycosubtilin [Yu et al. 2002, Gordillo et al. 2009]. According to Gordillo et al. (2009) a member of the iturins family, bacillomycin F inhibits the growth of *Aspergillus niger*, while iturin A inhibits the growth *A. flavus* and *Fusarium moniliforme.* Such a relationship may indicate a specific interaction between the pathogen and the metabolites of iturin.

In our work, inhibition of the mycelial growth of fungal species by the cell-free supernatants as sources of antifungal agents was also observed (Table 4 and Fig. 1 D-F). These results indicate an extracellular antimicrobial compounds produced by these species. Fungi appeared more sensitive towards supernatant obtained from the molasses broth cultures (Table 3). The inhibition was observed for *B. cinerea* A 258, *R. solani* W 70, *S. sclerotiorum* K 2291, *Phomopsis diachenii* K 657, *C. dematium* K 425, *P. complanata* A 233, *P. exigua var. exigua* A 175. However, the supernatants obtained from the brewery cultures inhibited mycelium growth of *Colletotrichum* dematium K 425 and *Phoma* species. Interestingly, *Phomopsis viticola* W 977, *Septoria carvi* K 2082 and *Colletotrichum gloeosporioides* A 259 were resistant to all tested supernatants. These results may be due to variable production of metabolites by *Bacillus* strains because Gordillo et al. [2009] demonstrated that culture media composition may also influence on the production of metabolites by *Bacillus* spp.

Previous investigations confirmed that the three *Bacillus* strains readily grew on different agricultural by-products (brewery effluents and molasses) as organic media (microbiological nutrients) and produced biosurfactants [Płaza et al. 2011]. Preliminary analysis of mass spectrometry with electrospray ionization and NMR (400 MHz) showed that the lipopeptides rich fractions of supernatants. Characteristic m/z peaks of

Table 3. Mycelium growth inhibition (in %) by the *Bacillus* strains growing in the different media
Tabela 3. Zahamowanie wzrostu badanych grzybów (w %) przez szczepy *Bacillus* rosnące w różnych podłożach płynnych

Phytopathogens Fitopatogeny	Mycelial growth inhibition (%) caused by *Bacillus* strains cultured in various media Zahamowanie wzrostu grzybów (%) spowodowane szczepami *Bacillus* hodowanych w różnych podłożach								
	T-1			T'-1			I'-1a		
	4	6	M	4	6	M	4	6	M
1. *Botrytis cinerea* A 258	62 ± 2.7	56 ± 2.2	41 ± 1.7	78 ± 1.4	63 ± 1.4	46 ± 0.8	62 ± 1.4	56 ± 0.5	47 ± 1.6
2. *Rhizoctonia solani* W 70	37 ± 1.4	2 ± 1.7	37 ± 2.0	26 ± 2.7	2 ± 1.1	40 ± 2.5	34 ± 0.9	16 ± 0.9	44 ± 1.2
3. *Sclerotinia sclerotiorum* K 2291	36 ± 0.9	27 ± 0.7	36 ± 1.6	40 ± 2.2	39 ± 0.9	33 ± 1.6	37 ± 1.2	45 ± 1.8	42 ± 1.1
4. *Phomopsis diachenii* K 657	31 ± 1.8	27 ± 1.8	31 ± 0.8	32 ± 1.7	31 ± 0.6	31 ± 1.2	35 ± 2.3	35 ± 1.1	32 ± 2.3
5. *Phomopsis viticola* W 977	31 ± 1.0	29 ± 2.5	51 ± 1.5	19 ± 1.0	38 ± 1.7	49 ± 2.4	31 ± 3.0	46 ± 0.9	54 ± 2.1
6. *Septoria carvi* K 2082	78 ± 0.7	39 ± 1.6	28 ± 1.2	67 ± 1.3	78 ± 1.3	31 ± 1.5	42 ± 1.4	64 ± 1.6	39 ± 0.9
7. *Colletotrichum dematium* K 425	19 ± 0.5	16 ± 0.9	32 ± 2.3	37 ± 2.2	51 ± 0.6	30 ± 0.9	14 ± 1.5	31 ± 1.1	41 ± 1.1
8. *Colletotrichum gloeosporioides* A 259	40 ± 1.4	52 ± 2.1	65 ± 1.7	46 ± 0.6	48 ± 1.5	62 ± 0.7	56 ± 0.7	48 ± 0.6	67 ± 2.6
9. *Phoma complanata* A 233	34 ± 1.2	54 ± 0.5	20 ± 0.6	52 ± 0.7	52 ± 0.9	30 ± 1.5	42 ± 1.7	46 ± 2.1	26 ± 1.2
10. *Phoma exigua* var. *exigua* A 175	43 ± 0.9	53 ± 2.4	27 ± 0.8	47 ± 1.1	57 ± 2.1	33 ± 1.6	43 ± 1.2	39 ± 0.8	33 ± 2.3

4, 6 – brewery effluents; M – molasses; Mean ± SD from three repeats;
4, 6 – ścieki browarnicze; M – melasa; Średnia ± odchylenie standardowe z 3 powtórzeń

Table 4. Mycelium growth inhibition (in %) by the cell-free supernatants obtained from the different cultures of *Bacillus* strains

Tabela 4. Zahamowanie wzrostu badanych grzybów (w %) przez supernatanty otrzymane z hodowli szczepów *Bacillus* rosnących w różnych podłożach płynnych

Phytopathogens Fitopatogeny	Mycelial growth inhibition (%) caused by cell-free supernatants of *Bacillus* strains growing on different media Zahamowanie wzrostu grzybów (%) spowodowane supernatantem z badanych szczepów *Bacillus* hodowanych w różnych podłożach								
	4			6			M		
	T-1	T'-1	I'-1a	T-1	T'-1	I'-1a	T-1	T'-1	I'-1a
1. *Botrytis cinerea* A 258	NI	7 ± 0.87	NI	6 ± 1.2	12 ± 0.93	9 ± 1.6	32 ± 2.03	34 ± 1.89	30 ± 1.55
2. *Rhizoctonia solani* W 70	NI	NI	NI	NI	NI	NI	17 ± 1.43	25 ± 0.98	27 ± 2.04
3. *Sclerotinia sclerotiorum* K 2291	NI	NI	NI	4 ± 02	NI	NI	18 ± 1.78	25 ± 2.89	26 ± 1.66
4. *Phomopsis diachenii* K 657	NI	NI	NI	25 ± 1.6	NI	NI	29 ± 0.89	22 ± 1.22	34 ± 1.09
5. *Phomopsis viticola* W 977	NI	NI	NI	NI	NI	NI	NI	NI	NI
6. *Septoria carvi* K 2082	NI	NI	NI	NI	NI	NI	NI	NI	NI
7. *Colletotrichum dematium* K 425	NI	NI	NI	30 ±2.1	9 ± 1.21	5 ± 0.4	12 ± 2.03	23 ± 1.99	14 ± 0.56
8. *Colletotrichum gloeosporioides* A 259	NI	NI	NI	35 ±1.3I	NI	NI	NI	NI	NI
9. *Phoma complanata* A 233	NI	NI	NI	28 ± 0.67	29 ± 1.03	31 ± 1.33	5 ± 1.03	9 ± 1.04	8 ± 0.45
10. *Phoma exigua* var. *exigua* A 175	NI	NI	NI	2 ± 0.62	27 ± 2.12	21 ± 0.98	22 ± 0.65	18 ± 1.22	25 ± 2.61

4, 6 – brewery effluents; M – molasses; Mean ± SD from three repeats; NI – no inhibition;

4, 6 – ścieki browarnicze; M – melasa; Średnia ± odchylenie standardowe z 3 powtórzeń; NI – brak inhibicji

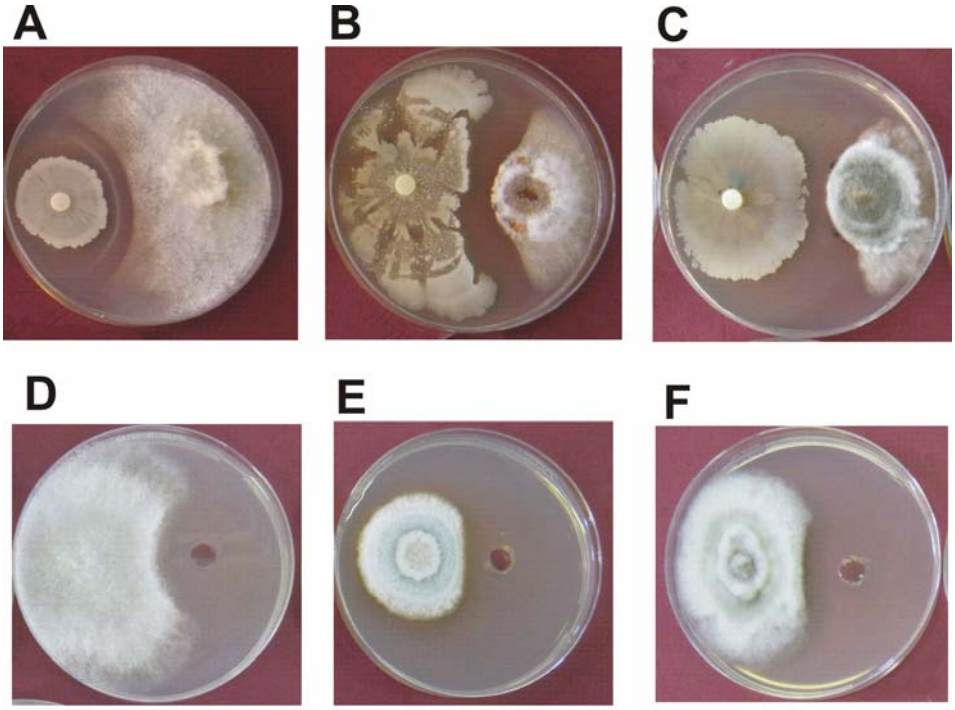

Fig. 1. Mycelium growth inhibition by *Bacillus* strains (A – C) and cell-free supernatants (D – F): A – inhibition of *Botrytis cinerea* A 258 by I'-1a strain growing on molasses; B – inhibition of *Colletotrichum gloeosporioides* A 259 by I'-1a strain growing on brewery effluents (# 6); C – inhibition of *Phoma exigua var. exigua* A 175 by T'-1 strain growing on brewery effluents (# 4); D – inhibition of *Botrytis cinerea* A 258 by T'-1 strain growing on molasses; E – inhibition of *Colletotrichum dematium* K 425 by I'-1a strain growing on molasses; F – inhibition of *Phoma exigua var. exigua* A 175 by T-1 strain growing on molasses

Rys. 1. Zahamowanie wzrostu wybranych gatunków grzybów przez szczepy *Bacillus* (A – C) i supernatanty otrzymane z ich hodowli (D – F): A – zahamowanie wzrostu *Botrytis cinerea* A 258 przez szczep I'-1a rosnący na melasie; B – zahamowanie wzrostu *Colletotrichum gloeosporioides* A 259 przez szczep I'-1a rosnący na ścieku browarniczym (#6); C – zahamowanie wzrostu *Phoma exigua var. exigua* A 175 przez szczep T'-1 rosnący na ścieku browarniczym (#4); D – zahamowanie wzrostu *Botrytis cinerea* A 258 przez szczep T'-1 rosnący na melasie; E – zahamowanie wzrostu *Colletotrichum dematium* K 425 przez szczep I'-1a rosnący na melasie; F – zahamowanie wzrostu *Phoma exigua var. exigua* A 175 przez szczep T-1 rosnący na melasie

surfactins, fengicyns and iturins families were observed in analysed samples [Poliwoda et al. 2012] The surface-active properties were most effective when *Bacillus* strains were cultured on molasses. The results obtained here and from previous experiments confirm that cell-free supernatants of *Bacillus* strains growing on agro-industrial wastes have antifungal activity probably caused by biosurfactants. Hence, the use of cell-free

culture supernatant directly to the evaluation of antifungal activity seemed to be more cost frugally than using pure biosurfactant.

Yoshida et al. [2001] described a potential antagonist of *Bacillus amyloliquefaciens* strain RC-2 against *Colletotrichum dematium*, mulberry anthracnose fungus isolated from mulberry leaves by *in vitro* and *in vivo* screening techniques. The culture filtrate of RC-2 also inhibited the growth of other phytopathogenic fungi and bacteria such as: *Rosellinia necatrix*, *Pyricularia oryzae*, *Agrobacterium tumefaciens*, *Xantomonas campestris pv. campestris*. The authors isolated and characterized antifungal compounds, and one of the compounds was determined as iturin A2. *B. amyloliquefaciens* is similar to *B. subtilis*, and it was distinguished from *B. subtilis* by the molecular ratio of G+C content in DNA [Yoshida et al. 2001]. Jacques (2011) described four main lipopeptides families from the *Bacillus* species: the surfactins, the iturins, the fengycins (or plipastatins) and the kurstakins. The toxicity of iturins to fungi has been found to rely on their ability to permeate membranes [Jacques 2011]. Also, fengycins have fungitoxic activity but more specifically against filamentous fungi. The fengycins action is less known compared to the other lipopeptides. Several studies present that lipopeptides are co-produced and active in synergistic way, for example surfactin with iturin, surfactin with fengycin and iturin with fengycin [Ongena et al. 2007, Jacques 2011].

Most of biosurfactants applications depend on their specific properties. In addition, several biosurfactants have been reported to have manifold biological activities like antibiotics, fungicides, insecticides, antiviral and antitumor agents or specific toxins and enzyme inhibitors. Developing knowledge of the biological properties is a key factor for introducing biosurfactants in high-added value products in different industries. Deleu and Paquot [2004] described specific activities of biosurfactants, especially in relation to their biological effects. Our results indicate that *Bacillus* strains growing on brewery effluents and molasses are also capable of antifungal activity against a wide variety of phytopathogenic fungi.

CONCLUSIONS

It has been shown here that low cost substrates such as brewery wastes and molasses may serve as growth media for these three *Bacilli* spp. that produce antifungal agents like biosurfactants. This bioprocess could be tested at a larger scale to produce enough material cost-effectively so such a project could be commercially viable. This could perhaps allow reduction of pesticide use and the associated environmental impact. The results here clearly indicate that these three *Bacillus* spp. growing on agro-industrial wastes and their cell-free supernatants as sources of biosurfactants have antifungal activities. Natural bacteria like *Bacillus* capable of suppressing pathogens and maintaining their population by competing against deleterious microorganisms could be successfully utilized as biopesticides for sustainable organic farming. Thus, these *Bacillus* species have the potential for application in biocontrol of plant diseases. Further investigations are currently conducted to isolate and characterize the pure biosurfactants and evaluate their efficacy as antifungal agents.

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WSTĘPNE BADANIA WŁAŚCIWOŚCI PRZECIWGRZYBOWYCH BAKTERII Z RODZAJU *Bacillus* ROSNĄCYCH NA ODPADACH Z PRZEMYSŁU ROLNO-SPOŻYWCZEGO

Streszczenie. Właściwości przeciwgrzybowe gatunków *Bacillus* hodowanych na ściekach browarnianych i melasie były oceniane. Bakterie wyizolowano z odpadów zanieczyszczonych związkami ropopochodnymi, a do ich identyfikacji wykorzystano następujące metody: amplifikację i sekwencjonowanie genu 16S rRNA, system Biolog oraz analizę kwasów tłuszczowych (FAME). W pracy stwierdzono, że bakterie *Bacillus* rosnące na ściekach browarnianych i melasie powodują zahamowanie wzrostu 10 badanych gatunków fitopatogenów, przy czym stopień zahamowania wzrostu był różny dla różnych gatunków grzybów. Najbardziej wrażliwe na badane bakterie były następujące gatunki grzybów: *Botrytis cinerea* A 258, *Phomopsis viticola* W 977, *Septoria carvi* K 2082, *Colletotrichum gloeosporioides* A 259, *Phoma complanata* A 233 and *Phoma exigua var. exigua* A 175. Jednocześnie stwierdzono również, że czyste supernatanty otrzymane z hodowli *Bacillus* na ściekach browarnianych i melasie powodują zahamowanie wzrostu grzybów na płytkach. Największą aktywność inhibicyjną wykazywały supernatanty otrzymane z hodowli bakterii *Bacillus* na melasie. Najbardziej wrażliwe na te supernatanty były następujące gatunki grzybów: *B. cinerea* A 258, *R. solani* W 70, *S. sclerotiorum* K 2291, *Phomopsis diachenii* K 657, *C. dematium* K 425, *P. complanata* A 233, *P. exigua var. exigua* A 175. We wcześniejszych pracach badano właściwości powierzchniowe supernatantów jako pośrednia metoda oceny obecności w nich biosurfakatntów. Szczepy *Bacillus* lub produkty przez nie syntetyzowane stanowią potencjalne źródło biopestycydów i mogą być wykorzystane w kontroli fitopatogenów, przyczyniając się do redukcji zastosowania chemicznych fungicydów.

Słowa kluczowe: *Bacillus* species, fitopatogeny, odpady z przemysłu rolno-spożywczego, biosurfaktant

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