Production of biosurfactants and antifungal compounds by new strains of Bacillus Spp. isolated from different sources

Received for publication, October 29, 2010
Accepted, February 5, 2011

1OLTEANU VIOLETA, 2SICUIA OANA, 3CIUCA MATILDA, 4CARSTEA DOINA MARIA, 5VOAIDES CATALINA, 1CAMPEANU GHEORGHE, 5CORNEA CALINA PETRUTA
1Center of Applied Biochemistry and Biotechnology (BIOTEHNOL), Bucharest, Romania
2Research and Development Plant Protection Institute – Bucharest, Romania
3National Agricultural Research and Development Institute Fundulea – Bucharest, Romania
4Institute of Biology – Bucharest, Romania
5Faculty of Biotechnology, USAMV Bucharest, Romania, email: pccornea@yahoo.com

Abstract

The identification of the ability of some new strains of Bacillus spp. isolated from Romanian soils to produce biosurfactants, the isolation of biosurfactants and evaluation their antimicrobial potential are the aim of this work. Six strains of bacilli were used in our experiments. Among them, five are new isolates, isolated from oil spilled soil (B1 and B2), from soil (BW) or from onion rhizosphere (OS15 and OS17). The strains B1, B2, BW and OS17 inhibited the growth of the majority of the fungal species tested in the experiments. The best results were obtained with B2 against Alternaria tenuis and OS17 against Fusarium oxysporum var. lycopersici. The ability to produce biosurfactants as antifungal agents was examined by oil spreading technique and emulsification stability test, using different types of oils: sunflower oil, olive oil, kerosene and kerosene mixed with 20% diesel. Differences in the ability of displace the oil and spread in the water and emulsifying oils were observed among the bacterial strains. The best results were obtained with the strains B2 and OS17 for all type of oils, but maximum action being recorded of kerosene. The microbial biosurfactants were isolated, detected by thin layer chromatography (TLC), and the antifungal action was also tested. The presence of genes for iturin production was also examined.

Key words: Biosurfactant, Bacillus subtilis, antifungal activity, molecular analysis, iturin.

Introduction

In plant-associated bacterial species, the production of biosurfactants is a common feature, and reflects the importance of these molecules for the functioning and survival of the producing organisms. Biosurfactants are biologically surface-active agents produced by various microorganisms such as bacteria, yeasts and fungi as membrane components or secondary metabolites [3, 11, 14, 15]. Biosurfactants are amphiphilic compounds, containing hydrophobic and hydrophilic moieties. Having both hydrophilic and hydrophobic domains, biosurfactants are able to partition at the water/air or water/oil interfaces and thus lower the interfacial of surface tension [8, 21].

In the last years, the biosurfactants produced by bacteria (both Gram negative and Gram positive bacteria) are getting more attention for their antifungal activity, owing their lower toxicity for plants and animals, high biodegradability, low irritancy and compatibility with human skin [2]. Also, this caused bioavailability insoluble in water substratum, to bind heavy metals, to interfere to sensing quorum process specific microorganisms and to form biofilms [17]. Among bacterial species, recent studies are focused on Bacillus genus for their ability to produced lipopeptides, a class of biosurfactants with antimicrobial effects. Lipopeptides are low molecular mass biosurfactants, including surfactin, iturin, lichenysins,
mycosubtilin, arthrofactin etc.), and exhibit surface-active properties, and antimicrobial activities [10, 11]. *Bacillus subtilis* produced a broad spectrum of bioactive lipopeptides with a great potential for biotechnological and biopharmaceutical applications [9].

The aim of this work was the characterization of some new strains of *Bacillus* spp. to produce biosurfactants and evaluation of their antifungal potential.

**Materials and methods**

**Microbial strains and culture conditions.** Six strains of bacilli and five strains of fungal pathogens were used in our experiments (table 1).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td><em>Bacillus subtilis</em> ATCC 6633</td>
<td>Faculty of Biotechnology Bucharest Collection</td>
</tr>
<tr>
<td>Bacteria</td>
<td><em>Bacillus</em> spp. B1</td>
<td>Isolated from oil spilled soil in the petroleum field, Ploiești area, Prahova</td>
</tr>
<tr>
<td>Bacteria</td>
<td><em>Bacillus</em> spp. B2</td>
<td>Isolated from oil spilled soil, Techirghiol, Constanța</td>
</tr>
<tr>
<td>Bacteria</td>
<td><em>Bacillus</em> spp. OS15</td>
<td>Isolated from onion rhizosphere</td>
</tr>
<tr>
<td>Bacteria</td>
<td><em>Bacillus</em> spp. OS17</td>
<td>Isolated from onion rhizosphere</td>
</tr>
<tr>
<td>Bacteria</td>
<td><em>Bacillus</em> spp. BW</td>
<td>Isolated from soil</td>
</tr>
<tr>
<td>Fungi</td>
<td><em>Alternaria tenuis</em></td>
<td>Isolated from tomato fruits</td>
</tr>
<tr>
<td>Fungi</td>
<td><em>Fusarium oxysporum</em> f. sp. <em>radicis lycopersici</em> ZUM 240</td>
<td>Dr. F. Constantinescu, IPP Bucharest</td>
</tr>
<tr>
<td>Fungi</td>
<td><em>Sclerotium bataticola</em></td>
<td></td>
</tr>
<tr>
<td>Fungi</td>
<td><em>Rhizoctonia solani</em></td>
<td></td>
</tr>
<tr>
<td>Fungi</td>
<td><em>Pythium</em> spp</td>
<td>Institute for Plant Protection Collection</td>
</tr>
</tbody>
</table>

Bacterial strains were maintained on LB agar slants. Fungi were maintained on potato dextrose agar (PDA).

The identification of bacterial isolates was performed both by microbiological methods, using API 20E and Biolog system, and by molecular methods. For molecular analysis, genomic bacterial DNA was isolated by Wizard R Genomic DNA Purification Kit. Restriction endonucleases Hae III and Msp I were purchased from Promega Corp. (Madison, Wis.) and the digestion of DNA was carried out as recommended the manufacturer. For PCR reaction universal primers f-ITS1 and r-ITS2 (Biosearch, Technologies INC.) were used. The sequences of f-ITS1 (5’-TCC GTA GGT GAA CCT GCG G-3’) and r-ITS2 (5’-GCT GCG TTC TTC ATC GAT GC-3’) were used. The amplification protocol consisted of one cycle of 94°C for 3 min, 38 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, and one cycle of 72°C for 10 min. The presence of iturin genes in bacteria was tested by using two primer pairs: for *itu*D (ituD-f: 5’-ATG AAC AAT CTT GCC TTT TTA-3’/ituD-r: 5’-TTA TTT TAA AAT CCG CAA TT-3’) and *lpa*-14 (lpa-14f: 5’-ATG AAA ATT TAC GGA GTA TA-3’) and lpa-14r: 5’-TTA TAA CAG CTC TTC ATA CG-3’)[11]. Multiplex PCR reaction was performed according to Hsieh et al.[9]. Amplification products were analyzed by electrophoresis in 1.2% agarose, in TBE (0,04M Tris-borate and 0,001M EDTA) buffer at 70V for 2 h.

Antifungal activity of bacterial isolates was examined by dual culture technique: agar blocks (5 mm dia.) containing 5-day-old mycelia were placed in the middle of PDA containing Petri plates and incubated for 24 h at 28°C. One loop of each bacterial culture was placed at 2.5 cm from the edge of fungal colony. Inhibition of mycelial growth was evaluated after incubation of 28°C for 5 days.
Biosurfactant production was evaluated after cultivation of bacteria in modified McKeen medium. The bacterial strains were inoculated in 100 ml of nutrient broth in a 250 ml of glass flask of screw cap (ISOlab, Germany) and incubated in a rotary shaker, 200 rpm at 30°C for 16-18 hours until cell numbers reach 10^6 CFU/ml. For biosurfactant production inoculums of 10% (v/v) was used. Bacterial strains were grown in 250 ml of glass flask of screw cap with 100 ml of modified McKeen medium (2.5% glucose, 0.25% monosodium glutamate, 0.3% yeast extract, 0.1% MgSO_4 x 7H_2O, 0.1% K_2HPO_4, 0.05% KCl and 0.1% (v/v) of trace elements solution (in 100 ml distilled water: 0.64 g MgSO_4 x 7H_2O, 0.16 g CuSO_4 x 5H_2O, 0.015 g FeSO_4 x 7H_2O); pH medium 7.0) and incubated in a rotary shaker, 190 rpm at 30°C for 96 hours (14). Biomass concentration was determinate during the cultivation of bacterial isolates in McKeen medium.

Oil spreading technique. 40 ml of distilled water were added to a large Petri dish (15 cm diameter) and 50 µl of oil (sunflower oil, olive oil, kerosene or kerosene mixed with 20% diesel) were placed to the surface of water. 10 µl supernatant (from culture broth) was added to the surface of oil. Occurrence of clear zone was an indication of biosurfactant production. The diameter of clear zone on the oil surface was measured and compared to 10 µl of distilled water as negative control [12].

Emulsification activity was measured using two methods: the measurement optical density at 540 nm [13] and the measurement emulsion stability after 24 hour through calculated emulsification index (E_{24}) [5]. In the first method, 2 ml samples of cell free supernatant were added to as screw-capped tubes containing 2 ml distilled water, and the solution mixed with 1 ml of a substrate (sunflower oil, olive oil, kerosene and kerosene mixed with 20% diesel). After a vigorous vortex for 2 min, the tubes were allowed to sit for 1 hour to separate aqueous and oil phase, before measuring the absorbance at 540 nm [11, 18]. Aqueous phase was removed carefully and OD at 540 nm was measured and compared with uninoculated broth used as negative control. Emulsification activity was defined as the measured optical density at 540nm [19]. Assays were carried out in triplicates. In the second method, 2 ml samples of cell free supernatant and 2 ml of oil were added to a screw cap tubes and vortex at high speed for 2 min. The mixtures were incubated at room temperature for 24 hours [15]. The emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixtures total height and multiplying by 100.

Extraction and analysis of bacterial biosurfactants. After 96 hours of cultivation, the bacterial cells were removed from 20 ml culture broth (in McKeen medium) by centrifugation at 5000 rpm for 10 min. 8 ml supernatants on bacterial isolates were acidified to pH 2.0 with 1N HCl and placed in a refrigerator at 4°C overnight. The pellets were obtained by centrifugation at 7000 rpm for 10 min and dissolved in 1.5 ml distilled water and the pH adjusted to 7.0 with 2M NaOH [14]. The crude biosurfactants were extracted as recommended Haddad et al. [7]. Extracts were examined by thin layer chromatography (TLC)[6, 11] and tested for antimicrobial activity.

Results and discussions

1. IDENTIFICATION OF BACTERIAL STRAINS

The bacterial strains were isolated from oil contaminated oil (B1 and B2), from soil and from onion rhizosphere (BW, OS15 and OS17 respectively). Staining technique, biochemical tests and molecular technique were performed in order to identify the bacterial isolates as Bacillus subtilis. The isolated strains, was found to be a Gram-positive, facultative anaerobe, motile, rod-shapes bacterium with a length of 1.7-2.3 µm. The bacilli Gram-positive were to identify with biochemical test Biolog-MicroLog on GP2 microplates. The
Production of biosurfactants and antifungal compounds by new strains of *Bacillus* Spp. isolated from different sources

metabolic profile was identified using database soft Microlog. According to these data, all the new isolates were identified as *Bacillus subtilis* strains. The confirmation of this conclusion was done by applying PCR technique [1, 17], using *B.subtilis* ATCC 6633 as reference strain. With ITS1-ITS2 primer pair unique amplicons with similar dimension were obtained (fig.1).

![Figure 1](image1.png)

*Figure 1.* PCR products obtained with universal primers ITS1 and ITS2. Lane 1, 123 bp molecular weight marker; lane 2, *B.subtilis* ATCC 6633; lane 3, B1; lane 4, B2; lane 5, BW; lane 6, OS15; lane 7, OS17

No difference between bacterial isolates tested (B1, B2, BW, OS15 and OS17) and the reference strain were observed after restriction analysis of PCR products previously obtained, using Hae III and Msp I restriction endonucleases (fig.2).

![Figure 2](image2.png)

*Figure 2.* Electrophoretic pattern of restriction products resulted after the cleavage of amplification products with Hae III (left side) or Msp I (right side) restriction enzymes. Lane 0, molecular weight marker; lane 1, *B.subtilis* ATCC 6633; lane 2, B1; lane 3, B2; lane 4, BW; lane 5, OS17; lane 6, OS15.

### 2. ANTIFUNGAL ACTIVITY OF BACTERIAL ISOLATES

Inhibition of the growth of some fungal pathogens by bacterial isolates was tested on PDA medium. The largest inhibition areas were observed with the strains B2 and OS17 against *A.tenuis*, *Pythium spp.* or *F.oxysporum* f. sp. *radicis lycopersici* ZUM 2407, respectively. No inhibition was observed against *R.solani* and *S.bataticola*.

For establishing the possible mechanism of inhibitory activity, both microscopic observations and molecular detection of specific genes involved in lipopeptide production were performed. The microscopic examination of the interactive zone allowed the observation of hyphal modification (vacuolization and cell disintegration) (fig.3).

![Figure 3](image3.png)

*Figure 3.* *A.tenuis* hyphal modification as result of antifungal activity of B2 strains
It is well known that the antifungal action of several bacilli is due mainly to the production of different lipopeptide, like iturin, surfactin, lichenysins, mycosubtilin etc. For this reason, the ability of the new bacterial isolates to produce iturin was checked by molecular techniques. Specific primers for gene involved in iturin A production were used in a multiplex PCR reaction. Among the six strains tested, clear amplification products, with expected length (1203 bp for \(ituD\) fragment and 675 bp for \(lpa\) fragment) were detected only in B2, BW and OS17 (fig.4). In \(B.subtilis\) ATCC6633 only the fragment corresponding to \(ituD\) gene was observed. No amplification products were obtained in B2 and OS15, suggesting that the iturin coding genes are not present or mutation in the region specific for the primers occurred.

![Figure 4](image)

Figure 4. Identification of \(ituD\) and \(lpa\)-14 genes by PCR. Lane 1, molecular weight marker; lane 2, \(B.subtilis\) ATCC 6633; lane 3, B1; lane 4, B2; lane 5, BW; lane 6, OS17; lane 7, OS15

Similar results were communicated for strains of \(B.amyloliquefaciens\) and \(B.circulans\) [9] confirming that the ability to produce iturin is a common feature among bacilli. The absence of amplicons in B2 suggests that the antifungal activity of this strain could be related to the production of other lipopeptides than iturin or to other antimicrobial compounds.

3. BIOSURFACTANT PRODUCTION

For biosurfactant biosynthesis the bacterial isolates were cultivated in Mckeen medium containing 2.5% glucose, at 30\(^\circ\)C in a rotary shaker at 190 rpm for 96 hours. The bacterial growth was evaluated by measuring OD at 600nm. It was shown that glucose increased both the biomass accumulation and biosurfactant production. Maximum growth level was found for B1, ATCC 6633, BW, and OS17 after 84 hours of incubation.

In order to detect the biosurfactant biosynthesis, oil spreading technique was used. As substrates, several types of oils were used: sunflower oil and olive oil), kerosene and kerosene mixed with 20% diesel. Differences in the ability of displace the oil and spread in the water was observed among the strains tested (table 2). The best results were obtained with the strains B2 and OS17 for all type of oils, but the maximum action was recorded on kerosene (zone formation of 28 mm and 30 mm, respectively.)

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Sunflower oil</th>
<th>Olive oil</th>
<th>Kerosene</th>
<th>Kerosene mixed with 20% diesel</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>4</td>
<td>5</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>B2</td>
<td>6</td>
<td>9</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>ATCC 6633</td>
<td>3</td>
<td>5</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>BW</td>
<td>3</td>
<td>6</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>OS15</td>
<td>3</td>
<td>4</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>OS17</td>
<td>5</td>
<td>9</td>
<td>30</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 2: Oil spreading technique for bacterial isolates tested.
Production of biosurfactants and antifungal compounds by new strains of \textit{Bacillus} Spp. isolated from different sources

Similar results were communicated in literature for different strains of \textit{B.subtilis} and \textit{Pseudomonas aeruginosa}: the strains were able to displace vegetable oil, kerosene, petrol and diesel [15].

The emulsification activity of the biosurfactants produced was tested through two methods: the measurement optical density at 540 nm of supernatants with different substrates (EA), and the measurement emulsion stability of supernatants with above noted substrates, after 24 hours (E$_{24}$). The highest emulsification activity was detected in B2 and OS17 against all types of substrates. It is of great interest that these bacterial strains have on increased emulsification activity against kerosene and kerosene mixed with 20% diesel.

Table 3: Emulsification activity of biosurfactants from bacterial isolates tested using different substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>B1</th>
<th>B2</th>
<th>ATCC 6633</th>
<th>BW</th>
<th>OS15</th>
<th>OS17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower oil</td>
<td>0.093</td>
<td>1.177</td>
<td>0.109</td>
<td>0.17</td>
<td>0.095</td>
<td>1.155</td>
</tr>
<tr>
<td>Olive oil</td>
<td>0.107</td>
<td>1.284</td>
<td>0.281</td>
<td>0.178</td>
<td>0.145</td>
<td>1.285</td>
</tr>
<tr>
<td>Kerosene</td>
<td>0.058</td>
<td>0.391</td>
<td>0.110</td>
<td>0.180</td>
<td>0.100</td>
<td>0.441</td>
</tr>
<tr>
<td>Kerosene mixed with 20% diesel</td>
<td>0.060</td>
<td>0.577</td>
<td>0.038</td>
<td>0.227</td>
<td>0.183</td>
<td>0.737</td>
</tr>
</tbody>
</table>

These results are comparable with those reported in literature: the emulsification activity (EA) of our isolates was similar to that of biosurfactant from \textit{B.subtilis} B6, where EA at 540 nm was 0.30 against kerosene [19]. Moreover, E.A. of biosurfactants from our isolate OS17 was superior to the reported values.

The emulsification stability (E$_{24}$) is another characteristic of biosurfactants and it was evaluated against the same substrates. The highest E$_{24}$ value was observed in OS17 (51.25% and 42.5% against kerosene and kerosene mixed with 20% diesel, respectively), comparing to B2 (48.75% and 41.25%, respectively) (table 4).

Table 4. Emulsification stability test for bacterial isolates tested

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Sunflower oil</th>
<th>Olive oil</th>
<th>Kerosene</th>
<th>Kerosene mixed with 20% diesel</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>30</td>
<td>27.5</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>B2</td>
<td>37.5</td>
<td>35</td>
<td><strong>48.75</strong></td>
<td><strong>41.25</strong></td>
</tr>
<tr>
<td>ATCC 6633</td>
<td>30</td>
<td>31.25</td>
<td>35</td>
<td>32.5</td>
</tr>
<tr>
<td>BW</td>
<td>32.5</td>
<td>33.75</td>
<td>43.75</td>
<td>36.25</td>
</tr>
<tr>
<td>OS15</td>
<td>30</td>
<td>31.25</td>
<td>41.25</td>
<td>35</td>
</tr>
<tr>
<td>OS17</td>
<td>40</td>
<td>32.5</td>
<td><strong>51.25</strong></td>
<td><strong>42.5</strong></td>
</tr>
</tbody>
</table>

The crude biosurfactants extracted were analyzed by TLC that revealed the presence of lipopeptides observed as red spot after spraying with ninhydrin reagent. The compounds produced by B2 and OS17 were compared with those synthesized by \textit{B.subtilis} ATCC6633 [5]. Similar results were observed by Priya et al. [15] and Fernandes et al. [6].

The biosurfactants extracted from B1, B2, \textit{B.subtilis} ATCC 6633 and BW were tested against \textit{A.tenuis} and \textit{S.bataticola} instead those extracted from OS15 and OS17 were tested against \textit{Fusarium oxysporum} f. sp. \textit{radicis lycopersici} ZUM 2407. After three days of incubation at 28°C, the compounds extracted from B2 and BW inhibited the growth of \textit{A.tenuis} (fig.5).
Figure 5. Antifungal activity of biosurfactants produced by B2 and BW against *A.tenius*

No inhibition was detected with extracts from OS15 and OS17. These results suggested that, at least for the strain OS17, the biosurfactants produced (including the lipopeptide iturin which coding genes were detected in this strain) had no inhibitory action against *F.oxysporum*. It is possible that the antifungal ability of this strain to be related to other compounds. However, the strain OS17 could be used for other applications (in bioremediation, for example), alone or in combination with other bacteria, including the bacilli tested in our experiments.

Similar results were reported in literature for lipopeptides isolated from *B.subtilis* 49 against the mycelial growth of *F.graminearum* and *Sclerotinia sclerotiorum* [16]. Cao et al. [3] had described the antibacterial and antifungal activities of a biosurfactant from *B.natto* TK-1. Moreover, the lipopeptide antibiotics producing by *B.subtilis* JA strongly inhibited phytopathogenic fungi, including *F.graminearum*, *R.solani*, *Pythium irregularare* and *Cladosporium fulvum* [4], and the surfactin produced by *B.mojavensis* RRC101, inhibited the mycelial growth of *F.verticillioides* [20].

Conclusions

The results obtained in our experiments allow several conclusions:
- By applying microbiological and molecular techniques, the new bacterial isolates were identified as *Bacillus subtilis* strains.
- All the isolates presented antifungal activity against various fungal phytopathogens, the presence of iturin coding genes being identified in some bacteria
- In the isolate B2 the presence of iturin coding genes was not detected, suggesting that the inhibitory action of the strains is related to other compounds
- The ability to produce biosurfactants was clearly detected in B2 and OS17 by oil spreading technique and by emulsification activity. The best emulsification activity and stability was observed in OS17 against kerosene and kerosen mixed with 20% diesel.
- The biosurfactants were extracted and analyzed, proving their antifungal action only in B2 and BW. Due to these action, the producing strains could be used, separately or in combination, in the biocontrol of some fungal pathogens
- No inhibitory action was detected in biosurfactants extracted from OS17. It is possible that the antifungal ability of this strain to be related to other compounds. The strain OS17 could also be used either for biocontrol or for other applications (in bioremediation, for example), alone or in combination with other bacteria, including the bacilli tested in our experiments.

Aknowledgements

The research was partially supported by PNCDI II Research Program, project no.204-2008 (acronym CEMAGRIM)
References


