Selection Of An Antifungal *Bacillus niabensis* From Algerian Salt Soil And Study Of Its Potential Of Surfactin Production

Mounia Youcef-Ali¹, Noreddine Kacem Chaouche², Laid Dehimat³, Asma Ait Kahi⁴, Jacqueline Destain⁵ and Philippe Thonart⁶.

¹,²,³,⁴ Laboratory of Mycology, Biotechnology and Microbial activity, FSNV, University Constantine-1 Constantine, 25000 (Algeria).

⁴,⁵,⁶ Walloon Centre for Industrial Biology, Agro-Biotech, University of Liege Gembloux, 5030 (Belgium).

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*Bacillus niabensis* CWBI-B1569 (accession number KC341750) was isolated from salt soil of two different saline regions of Algeria and screened for its production of bioactive compounds. Both the antagonistic and cell-free culture supernatant of strain CWBI-B1569 showed strong *in vitro* growth inhibition activity against *Aspergillus repens*, *Candida albicans*, *C. tropicalis* and *Yarrowia lipolytica*. Bioactive molecules were produced by *B. niabensis* CWBI-B1569 in a liquid culture medium optimised for lipopeptide production. Inhibition of fungi was equally demonstrated by testing the resulting supernatant and Lipopeptide-enriched extracts. The association of mass spectrometry and liquid chromatography (LC-MS) analysis revealed the presence of compounds which were similar to the lipopeptide Surfactin. Surfactin purified from *Bacillus niabensis* CWBI-B1569 had three homologous, C₁₄, C₁₅ and C₁₆, with protonated masses of m/z 1044.7, 1058.7, and 1072.7, respectively. Thus, *B. niabensis* CWBI-B1569 may play an important role in biological control, and should be added to the list of *Bacillus* species, as one of the largest sources of bioactive natural products.

**Keywords:** *Bacillus niabensis* CWBI-B1569, antifungal activity, Surfactin, LC-MS, saline lake.

**INTRODUCTION**

Bioactive natural products constitute a library of compounds with a large and privileged structural diversity, showing a variety of biological activities. In fact, many of them have been used for pharmaceutical applications (Salas et al., 2007). In the course of screening for new antibiotics, several research studies are currently oriented towards the isolation of new microorganism species from different soils and ecosystems (Melloul, et al., 2003). Inside microorganisms, several species of *Bacillus* genus are able to produce biologically active substances that are capable of disintegrating the fungal cell walls (Tserkovniak et al., 2009). In the midst of these substances, several lipopeptide compounds, such as Surfactin, Iturin and Fengycin, serve as a class of microbial surfactants with increasing scientific, pharmaceutical and biotechnological interest (Ongena, and Jacques, 2008).

Surfactin is an important biosurfactant with superior surface activity and belongs to a group of cyclic lipopeptapeptides containing beta-hydroxyl fatty acids and D2/L amino acid residues (Haddad et al., 2008). Surfactin has numerous environmental and biotechnological applications (Solaiman, 2005) and has shown particular utility in oil recovery (Schaller et al., 2004), remediation of soil contaminated by heavy metals (Zouboulis et al., 2003), biocontrol against phytopathogens (Ongena, and Jacques, 2008) and

*Corresponding author. E-mail: mouniayoucefall@yahoo.fr.*
An important characteristic of this compound is its ability to lyse red blood cells and may act as an antibiotic, antiviral and haemolytic agent (Carrillo et al., 2003).

Surfactins are mainly composed of three components: C13-surfactin, C14-surfactin, and C15-surfactin. Of those, C15-surfactin has the highest surface activity, about 1000 times higher than the traditional chemical surfactant sodium dodecyl sulphate (SDS) (Yoneda et al., 2001; Razafindralambo et al., 2004), and haemolytic activity (Kracht et al. 1999). C15-surfactin also has other activities, including anti-tumour, antimicrobial, anti-mycoplasma functions, and is an efficient synergistic antifungal agent (Cao et al., 2009; Cao et al., 2010; Liu et al., 2012). Due to its amphiphatic nature, surfactin incorporates into the phospholipid bilayer and induces permeabilisation and perturbation of target cells. The rising antibiotic resistance, as well as a number of remarkable surfactin activities, shows that it deserves special interest and is considered a candidate compound for combating several health related issues (Seydlová and Svobodová, 2008).

In fact, the list of novel microorganisms, especially Bacillus, and products found in microbiologically unexplored ecosystems around the world suggest that a careful exploration of other habitats such as saline regions might continue to be useful. The Algerian Salt Lake presents an ecosystem with numerous opportunities as a source of novel microorganisms producing new compounds.

To our knowledge, B. niabensis has not yet been isolated from soil and no studies have been performed concerning its antifungal activity, and its potential of lipopeptide production. Nevertheless, in this work, we have tried to demonstrate the antifungal activity of Bacillus niabensis CWBI-B 1569 and its potential for Surfactin production. This strain was isolated from soil of two different saline lakes in East and South East of Algeria.

**MATERIALS AND METHODS**

**Isolation of Bacterial strains**

Soil samples taken from two saline lakes, situated in Eastern Algeria, were studied (Table 3). One gram of each sample was suspended in 9 mL sterile physiological water and shaken vigorously for 2 min. The soil suspension was serially diluted in sterile physiological water (from $10^1$ to $10^6$); all dilutions were plated on YPD medium (Glucose, 20 g; Peptone, 10 g; Yeast extract, 10 g; Agar, 15g; distilled water, 1 L) supplemented with a commercial antifungal to inhibit mould growth. Petri dishes were incubated at 30°C (Gerhardt, 1994). The bacterial strains were maintained on YPD medium at 4°C before experimental use, and stored at -80°C in cryotubes according to the manufacturer recommendations (Microbank, Prolab Diagnostic, Richmond Hill, Canada) for long-term storage.

**Screening of antagonists strains by in vitro antagonism experiments**

The antifungal activity of the isolates was determined according to the plate diffusion method (Errakhi et al., 2007) against Aspergillus repens, Candida tropicalis, C. albicans and Yarrowia lipolytica.

C. albicans (provided by the Laboratory of Parasitology and Mycology, CHU Constantine, Algeria), C. tropicalis and Yarrowia lipolytica (Walloon Centre for Industrial Biology) were maintained on Yeast Malt Agar (YMA: Glucose, 20 g; Peptone, 10 g; Yeast extract 10 g; Agar, 15 g; distilled water 1L) containing Gentamycin and Chloramphenicol, while Aspergillus repens (Laboratory of Mycology, Biotechnology and Microbial Activity, University Constantine 1, Algeria) was maintained on Sabouraud (SAB: Glucose, 20 g; Casein peptone, 5 g; Gelatine peptone, 5 g; Agar, 15 g; distilled water 1L). Isolates were grown on YPD medium for 24 h and discs (6 mm in diameter) were cut and placed on YMA and SAB mediums; the first was seeded by spreading of the yeast suspensions, while the second was seeded by spreading the sporal suspension of filamentous fungi. Plates were first kept in a refrigerator (4°C) for at least 2 h to allow the diffusion of any antibiotics produced, then incubated at 30°C. Inhibition zones were determined after 24 h and 48 h of incubation for yeast and after 3 and 4 days for fungi. The same method was used to test the antagonistic effect of cell-free culture supernatant. In this case, 80µL aliquots of filter (0.2µm) sterilised supernatant samples were dispensed in wells (performed with a sterile cork borer, of 6mm diameter) made in the gelified medium previously spread by the fungal suspension (Touré et al., 2004).

Among the strains which developed antifungal activity, one bacterial strain (coded as CWBI-B1569) was selected for further study and screened for lipopeptide production.

**Characterisation of the selected antagonistic bacteria CWBI-B 1569**

To identify the strain CWBI-B 1569, Gram staining, a catalase test, morphology and standard biochemical tests, as listed in Bergey’s Manual of Determinative Bacteriology, and their ability to utilise various carbon sources (elements) in the API 50CH (Biomerieux) were assayed (Logan and Berkeley, 1984). The selected antagonistic bacterial strain was also identified by sequencing of its 16S rDNA.
Sporulation test

The strain CWBI-B 1569 was grown in a liquid culture medium (YPD: Glucose, 20 g; Peptone, 10 g; Yeast extract, 10 g; distilled water, 1 L) at 30°C for 3 days in agitated flasks at 200 rpm. The cells were examined by a specific microscope for observation of spores: Zeiss Axioskop 2 MOT, Germany.

Thermal treatment

Strain CWBI-B 1569 was inoculated into nutrient broth and incubated with shaking for 24 h at 30°C. The cultured sample was diluted into a minimal medium salt base, and the cell suspension was incubated for 12 min at 80°C. Samples of treated and untreated cultures were diluted and plated on Nutrient Agar for CFU counting.

Identification by sequencing 16S rDNA gene

The Wizard ® genomic DNA purification kit (Promega, Madison, USA) was used to isolate the total DNA of the strain CWBI-B1569. PCR amplification was performed with the primer pair SPO/SP6 (5'-AAGAGTTTGATCTCGTCAAG-3'/5'-CTACGCTACTTGTAGCTCA-3') targeted against regions of 16S rDNA (Ventura et al., 2001) DNA amplification was carried out in a Master Cycler Personal (Eppendorf) and the following programme was used: initial denaturation for 5 min at 96°C, 25 cycles of hybridisation (30 sec at 55°C), polymerisation (2 min at 72°C), elongation (30 sec at 95°C) and extension (10 min at 72°C). The amplified products were resolved in a 1% (w/v) agarose gel electrophoresis in TAE buffer AND visualised by ethidium bromide staining.

Salt tolerance

Since the strain CWBI-B 1569 was isolated from a hypersaline environment, salinity tests were performed to determine the concentrations of NaCl tolerated by the strain CWBI-B1596. For assays of salt tolerance, bacterial cultures of the selected strain were incubated in nutrient broth supplemented with different concentrations of NaCl (0 g/L, 20 g/L, 40 g/L, 60 g/L, 80 g/L, and 100 g/L). The growth was monitored after 72 h incubation at 30°C.

Production and purification of lipopeptide

Strain CWBI-B1596 was grown at 30°C for 72 h in agitated flasks at 200 rpm in 350 ml of a liquid culture medium optimised for lipopeptide production (named Opt medium) and described by Jacques et al. (1999). Cultures were centrifuged at 17,400 g for 20 min. The supernatant was filtered through a 0.2 µm sterile filter membrane for testing by the plate diffusion method against fungi.

In order to obtain lipopeptide-enriched extracts, purification of the resulting supernatants was realised by solid-phase extraction of 300 mg on a C18 column (Touré et al., 2004); this is sufficient to fraction 10-20 mL of classic supernatant (agitated culture of 3 days in rich medium).

Ten millilitres of crude supernatant was loaded on an ISOLUTE C18 CE type cartridge (International Sorbent Technology) previously activated with 10 mL of methanol and equilibrated with 5 mL of Milli-Q water. The column was washed with 3 mL of Milli-Q water. Hydrophobic compounds were eluted with 1 mL of 100% MeOH. This methanolic supernatant extract was also tested.

Characterisation and identification of lipopeptides using LC-MS

Lipopeptide-enriched extracts prepared as described above were analysed using a reverse phase HPLC (HPLC Waters Alliance 2695/ diode array detector) coupled with a single quad mass spectrometer (Waters S-QD mass analyser) on an X-terra MS (Waters) 150*2.1 mm, 3.5 µm C18 column. A diode array detector was integrated into the HPLC system. The flow rate and temperature of the column were set at 0.5 mL/min and 40°C, respectively. The elution gradient mode was used to separate the various homologous compounds (Table 1). The ionisation type was electrospray. Desolvation and source temperatures were 250°C and 130°C, respectively. Nitrogen flow was set at 500 l/h. Elution solvents were acetonitrile and Milli-Q water, with 0.1% formic acid. Specific cone voltages of 70, 85 and 130 V were used for surfactins, iturins and fengycins, respectively. The positive ion mode was used for analysis of all three families because a higher signal/background
Table 1. Total flow rates, concentrations of the solvents used and their times

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (H2O 0.1 FA)%</th>
<th>Solvent B (ACN 0.1 FA)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>18</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>19</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>24</td>
<td>55</td>
<td>45</td>
</tr>
</tbody>
</table>

ACN: Acetonitrile, FA: Formic acid

Table 2. Bacterial strains obtained from salt soils of Melghigh and Ain M'Lila lakes.

<table>
<thead>
<tr>
<th>Localities</th>
<th>Sample’s nature</th>
<th>Number of samples</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melghigh</td>
<td>Soil lake</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Ain M’Lila</td>
<td>Soil lake</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Soil near the lake</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Rhizosphere</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

RESULTS

Isolation of bacterial strains

For the selection of a potential antagonist which was able to inhibit fungal growth, over 50 bacterial isolates were obtained from the salt soil of two different saline regions situated in the East and South-East of Algeria (Table 2).

Screening of antagonists by in vitro antagonism experiments

In order to assess whether the bacterial strains isolated may be useful sources for natural bioactive compounds, the antifungal activity of the isolates and their supernatants was determined. Among these isolates, six strains showed an antagonistic effect when faced with: Aspergillus repens, Candida albicans, C. tropicalis and Yarrowia lipolytica in the in vitro assay. However, only one strain, coded CWBI-B1569 was selected to be carefully studied. This strain showed particularly strong antifungal activity.

Identification of the strain CWBI-B 1569

Upon microscopic examination, catalase activity and the Gram stain showed that strain CWBI-B1596 was catalase and Gram positive. Sporulation tests, thermal treatment and identification made by API 50CH revealed that this strain belongs to the genus: Bacillus. However, nucleotide sequences of 16S rDNA of the Bacillus CWBI-B1569 were assessed to confirm biochemical identification. The 16S rDNA sequences of this strain was determined and compared with available 16S rDNA sequences in the GenBank database. The sequences were similar to each other (>99% similarity). This comparison showed that the strain CWBI-B1569 was Bacillus niabensis (Figure 1). The 16S rDNA gene sequence of the strain was submitted to the GenBank database with the accession number KC341750.

Description of Bacillus niabensis CWBI-B 1569

Cells are Gram-positive, motile and spore-forming. Colonies are yellowish white, 2–3 mm in diameter and circular, with clear margins after incubation on YPD medium for 2 days. Growth occurs at 30°C and at pH 7.0.

Salt tolerance

The halotolerance of B. niabensis CWBI-B 1569 was proven by salinity test. The results show that Bacillus niabensis can grow in high concentrations of NaCl, reaching even 80 g/L; furthermore, good development of the strain was observed at 40 g/L of NaCl.

Production and purification of lipopeptides

As lipopeptides of the Iturin, Fengycin and Surfactin groups are among the most bioactive non-polar antibiotics produced by several strains of Bacillus genus, their production by Bacillus niabensis CWBI-B 1569 in the Opt medium was studied here. However, the production of bioactive molecules with antifungal activity
Figure 1. Phylogenetic relationships of strain CWBI-B 1569 (coded by the database as $\text{d}[5+123]$ and other closely related Bacillus sp. based on their 16S rDNA gene sequences.

Figure 2. Inhibition of Y. lipolytica by filter-sterilisation of the crude supernatant of B. niabensis CWBI-B1569 after growth for 72 h in Opt medium. The synthesis of lipopeptides (compounds probably responsible of the antifungal activity) was further exemplified by testing filter-sterilised crude supernatants obtained from culture in the Opt medium. For safety, these tests were performed against Y. lipolytica. A total of 80 µL of this solution was sufficient to clearly inhibit the development of yeast (Figure 2). A similar level of antagonistic activity was observed by testing lipopeptide-enriched extracts, suggesting the involvement of secreted antifungal metabolites (Figure 3).
Characterisation and identification of lipopeptides using LC-MS

Only one of the different families of lipopeptides was detected in the methanolic extract obtained from the culture supernatant. This was identified by HPLC-MS as Surfactin (Figure 4, 5) on the basis of the retention times and corresponding peak areas compared with purified molecules used as standards. A wide variety of homologous compounds were detected, including Surfactin C_{14} to C_{16} (Table 3 and Figure 6).

DISCUSSION

Soil microorganisms constitute the world’s largest reservoir of biological diversity; the *Bacillus* genus represents a large number of microorganisms commonly
Table 3. Surfactin homologues produced by *Bacillus niabensis*. Compounds were identified by the combination of mass spectrometry and HPLC on the basis of their retention times compared with purified standards.

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Area</th>
<th>Surfactin homologous</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.474</td>
<td>260193,016</td>
<td>C_{14}</td>
</tr>
<tr>
<td>14.286</td>
<td>230925,094</td>
<td>C_{15}</td>
</tr>
<tr>
<td>14.844</td>
<td>507608,938</td>
<td>C_{16}</td>
</tr>
</tbody>
</table>

Figure 6. HPLC/MS m/z corresponding to the three homologues of Surfactin: C_{14}, C_{15}, C_{16}, with their protonated masses of 1044.7, 1058.7, and 1072.7, respectively.
isolated from soil, which are considered to be safe microorganisms and to possess the remarkable abilities of synthesising a vast array of beneficial substances (Liu et al., 2010). In that context, the isolation of new bacterial strains with promising biocontrol potential is necessary to enlarge the still limited range of marketable products. Our research has led to the selection of a Bacillus strain, coded as CWBI-B1569, originating from saline regions and identified as Bacillus niabensis. This strain has an antifungal power, especially against C. tropicalis, C. albicans, Y. lipolytica and A. repens. To our knowledge, Bacillus niabensis has not been previously isolated from salt soil and no studies have been performed concerning its antifungal activity, especially with regard to the production of a lipopeptide group.

Results presented in this article show the capacity of Bacillus niabensis CWBI-B1569, isolated from salt soils originating from Algerian saline regions, to inhibit the growth of various fungi and its potential secretion of one group of lipopeptides, identified as the Surfactin group. Previous studies have described microbial population and Bacillus diversity in saline soils (Das et al., 2008). Among the bacterial isolates obtained in our study, strain CWBI-B1569 showed a powerful antifungal activity; both the antagonistic and cell-free culture supernatant significantly inhibited growth of the test fungi. However, Ben Maachia et al. (2010) studied antifungal and antibacterial activities of Bacillus sp. strains isolated from salt soil in Tunisia. It is well known that several species of Bacillus are capable of producing biologically active substances, including antifungal substances; among the Bacillus species most studied are: B. subtilis (Touré et al., 2004; Yun-Feng et al., 2012), B. vallismortis (Zhenzhen et al., 2010), B. pumilus (Cho et al., 2009) and B. amyloliquificiens (Caldeira et al., 2011). Based on the physiological and biochemical characteristics and 16S rDNA sequence analysis, strain CWBI-B1569 was identified as B. niabensis. To the best of our knowledge, the antifungal power of Bacillus niabensis has not yet been studied. Besides, this species was discovered recently for the first time by Kwon et al. (2007) and was named B. niabensis, (niab.en’sis. N.L. masc. adj. niabensis arbitrary name formed from NIAB, the acronym for the National Institute of Agricultural Biotechnology, Korea). Subsequently, it was implicated in the degradation of benzylidimethyl hexadecylammonium chloride by Bassey and Grigson, (2011).

In this study, colonies of Bacillus niabensis CWBI-B1569 were yellowish white, 2–3 mm in diameter and circular with clear margins on YPD. Its growth occurred at 30°C and at pH 7.0, which corroborates the results of Kwon et al. (2007). Furthermore, B. niabensis can tolerate up to 80 g/L of NaCl, which confirms its halophilic character. The same author showed that the NaCl range growth of B. niabensis was in the range of 0-5%. Antifungal power of B. niabensis was further exemplified by testing filter-sterilised crude supernatant obtained from its culture in the Opt medium. The bioactive compounds of the culture filtrate were purified and identified using LC-MS as Surfactins with three homologous: C14, C15 and C16. It has been recently reported that C14-Surfactin and C15-Surfactin had synergistic antifungal activities with ketoconazole (KTC) against Candida albicans. C15-Surfactin as a biomaterial could be utilised as a synergistic antifungal agent with KTC for novel applications in biomedical and pharmaceutical fields (Liu et al., 2012); this study also offered a novel marine derived B. amyloliquificiens strain MB199, which could efficiently produce C15-surfactin in shaker flasks.

Hassan et al. (2010) found that some Bacillus species produce Surfactin lipopeptide, hydrolytic enzymes and other secondary metabolites which had strong antifungal activity against Colletotricum falcatum. Surfactin has been known to interact with the cell membrane and disturbs the membranes stability (Eeman et al., 2006). In addition, Maget-Dana and Ptak, (1995) have also reported that Surfactin has synergistic activity with iturin on its haemolytic activity. However, there is limited knowledge about the antifungal and synergistic antifungal activities of Surfactin lipopeptide.

Nevertheless, through this study, we have tried to demonstrate the antifungal activity of Bacillus niabensis CWBI-B 1569 and its potential for Surfactin production. Thus, B. niabensis CWBI-B1569 and its bioactive component may play an important role in biological and pharmaceutical control. It should be added to the list of Bacillus species, one of the largest sources of bioactive natural products. Moreover, the efficacy of Surfactin as a potential antifungal compound should be further studied.

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