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# Antifungal agent production from a new marine *Bacillus pumilus*SMH101

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**Twenty two (22) marine bacterial strains were isolated and tested to inhibit some plant and human pathogenic fungi; *Fusarium solani*, *Rhizoctonia solani, Aspergillus niger*, *Fusarium exosporium* and *Candida albicans*. The most potent marine bacterium was identified as *Bacillus pumilus*SMH101 on using 16S rRNA. The peptone water medium (PW) showed maximum antifungal activity. The Placket-Burman experimental design was applied and the optimum culture conditions were inoculum size (8.0 × $10^6$ cfu/ml), temperature (25°C), incubation period (24 h) and pH value of 7.0. The trickle flow column was tested for propagating the antifungal production using luffa pulp and synthetic sponge as solid packing materials. The adsorbed *B. pumilus*SMH101 showed lower average fungal suppression (~ 40%) compared to the free bacterial cells (58.6%). Thin layer chromatography (TLC) was applied using a solvent system of dichloromethane: methanol: water (65:32:3 v/v). A single ultraviolet (UV) spot was obtained with a retardation factor ($R_f$) of 0.75. It analyzed using UV, infrared (IR) and mass spectrometry (MS) spectra and showed a molecular weight of 875 Da. Also, it showed a relatively low bio-toxicity ($LC_{50}$ = 1072 ppm) and a broad antifungal spectrum with a bioactivity of 87, 80 and 70% against *F. solani*, *R. solani* and *C. albicans*, respectively, compared to some commercial antifungal drugs based on the active fluconazole compound which applied in a concentration of tenfold more than the used *B. pumilus*SMH101 antifungal agent concentration (0.05 mg/ml).**

**Key words:** Antifungal, optimization, Placket-Burman, trickle flow column, *Bacillus pumilus*.

## INTRODUCTION

In recent years, the incidence of invasive opportunistic fungal infections has been increasing due to increases in the number of immune-compromised patients (Soeta et al., 2009). Infections due to *Candida* species in immune-compromised patients are the most common; however, *Aspergillus* and other pathogenic fungi are also emerging as a threat to public health (Richardson, 2005). The mor-tality rate due to invasive aspergillosis has risen stea-dily with a 35.7% increase which caused significant mor-bidity and mortality during 1980 to 1997 (Rapp, 2004). Infecions due to *Fusarium* species are emerging hyalo- hypho-mycoses of immune-compromised patients and are asso-ciated with high mortality (Nucci and Anaissie, 2006). The dismal prognosis of *Fusarium* infection is the result of

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limited therapeutic options. Recent reports suggest that *Fusarium* species not only affect bone marrow transplant recipients and patients with haema-tological malignancies (Nucci et al., 2004), but also solid-organ transplant patients. In some centers, *Fusarium* species are the second most common cause of mold infection after *Aspergillus* species. The *Fusarium* species most frequently implicated in human infections include *Fusarium solani, Fusarium oxysporum* and *Fusarium moniliforme* (Lisboa et al., 2006).

*Candida* species belong to the normal micro-biota of an individual's mucosal oral cavity, gastrointestinal tract and vagina and are responsible for various clinical manifestations from muco-cutaneous overgrowth to blood-stream infections (Eggimann et al., 2003). Moreover, fungal infections have increased since the 1980s, especially in the large population of immune-compromised patients and/or those hospitalized with serious underlying diseases (Espinel-Ingroff et al*.,* 2009; Sardi et al*.,* 2013).

Recently, marine bacteria are being recognized as an important resource for many antimicrobial products, especially antifungal activities (Woo et al., 2002). Moreover several researchers extracted bioactive substances from different bacteria and actinomycetes, which exhibited biological activity against many pathogenic fungi; *Lactobacillus plantarum* (Storm et al*.,* 2002), *Chromobacterium violaceum* (Barreto et al., 2008), chitinolytic marine strains (Gohel et al., 2004), *Kitasatospora* sp. (Haesler et al*.,* 2008), *Streptomyces malaysiensis* (Li et al*.,* 2008), *Streptomyces* sp. (Park et al*.,* 2008). Also, some marine *Bacilli* were used for antifungal production such as *Bacillus firmus* (Ortega et al., 2009), *Bacillus megaterium* (Kong et al., 2010).

The application of statistically based experimental designs is a must to deal with the effect of different culture variables in the same time (Ooijkaas et al*.,* 1998). The Plackett-Burman designs are powerful tools for searching the most significant factors for antimicrobial production (Bie et al., 2005).

Immobilization techniques mostly applied for the production of secondary metabolites through cell entrapment and cell adsorption on solid supporters (Asanza et al*.,* 1997). The application of immobilized cells to study microbial processes is one of the main trends in modern biotechnology. In addition, cell immobilization shows many operational and economic advantages such as prolong metabolic activities, reuse of the biocatalyst, increase of cell concentration and preventing washing out of cells when high flow rates were applied (Gautam et al*.,* 2002).

In this study, a new marine *Bacillus* strain was isolated and identified using 16S rRNA as *Bacillus pumilus*SMH101. It was tested to produce antifungal agent acting against some plant and human pathogenic fungi. The optimization for antifungal production was carried out using Plackett-Burman experimental design as, the immobilization technique. A partial purification and characterization of the produced antifungal agent was carried out and the $LC_{50}$ of the bioactive spot was determined using *Artemia salina* as a biomarker.

## MATERIALS AND METHODS

### Pathogenic fungi

The pathogenic *Aspergillus niger, Fusarium solani, Penicillium oxilacum***,** *Rhizoctonia solani* and *Candida albicans* ATCC 14053 were kindly provided from Dr. Eman AbdEl-Zaher, Tanta University, Egypt. The maintenance of these fungi except *C. albicans* was carried out using a modified Czapek Yeast Extract Agar (CYEA) medium, it Composed of (g/L): Sucrose, 20; $NaNO_3$, 2.0; $K_2HPO_4$, 1.0; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; Yeast extract, 2.0. The pH was adjusted at 5.0 (Atlas, 2010). While, *C. albicans* was maintained using Sabouraud-dextrose agar (SDA) medium (British Pharmacopoeia) according to Sandven and Lassen (1999).

### *Isolation and purification of antifungal producer(s)*

Seawater samples were collected from different locations in Alexandria shore line using 500 ml sterile blue screw-caped bottles according to the standard methods published by American Public Health Association (APHA, 1995). Serial dilutions were made using filtered sterilized seawater (from $10^{-2}$ to $10^{-6}$). A portion (0.1 ml) from each diluted sample was spread on seawater nutrient agar plate medium (5 g peptone, 3 g beef extract, 20 g agar, 1000 ml seawater). Plates were incubated at 30°C for 24 h. A purification of the obtained bacterial colonies was carried out by streaking technique. The purified bacterial colonies were kept separately and tested to inhibit the pathogenic fungi; *A. niger, F. solani***,** *P. oxilacum, R. solani* and *C. albicans* ATCC 14053.

### Bioactivity test

The bioactivity of each bacterial isolate against the pathogenic fungi was estimated as follows; a suspension of 24 h old bacterial culture (OD ~ 1.0) was centrifuged at 12000 rpm for 10 min. Then 5 ml of each bacterial supernatant was added to 100 ml (CYEA) medium and poured using 9 cm sterile Petri dishes (amended (CYEA) plates). 0.5 cm cylinders with 5 mm diameter were cut out from pre-activated pathogenic fungi using a sterile cork borer, and then transferred separately to the center of the amended (CYEA) plates and the control (un-amended (CYEA) plates); all plates were incubated at 28°C for a week. The suppression percentage was calculated as follows: (fungal growth diameter on un-amended (CYEA) plates in mm - fungal growth diameter on amended (CYEA) plates in mm)/ fungal growth diameter on un-amended (CYEA) plates in mm × 100% (APHA, 1995).

While, the bioactivity against *C. albicans* was carried out as follows: 5ml of each bacterial supernatant was added to 100 ml (SDA) medium and poured using 9 cm sterile Petri dishes (amended (SDA) plates). 100 µl of *C. albicans* suspension (OD ~ 1.0) was added to amended (SDA) and the control (un-amended (SDA) plates).

All plates were incubated at 30°C for 48 h. The suppression percentage was calculated as follows: (No. of colonies on un-amended (SDA) plates in cfu/ml - No. of colonies on amended agar plates in cfu/ml)/ No of colonies on un-amended (SDA) plates × 100% (APHA, 1995).

**Molecular identification process**

This process was carried out at National Research Institute, El-Doky, Cairo, Egypt.

**DNA extraction**

Total DNA content was extracted from overnight pure culture of the most bioactive marine bacterial isolate using Qiagen DNeasy kit (QIAGEN-Inc., Germany) and Genomic DNA purification kit (Promegal). The procedure was identical to that recommended by the manual instructions. The preparations were analyzed on a 0.7% agarose gel and then determined spectrophotometry (Sambrook et al., 1989).

**Polymerase chain reaction (PCR)-amplification and sequencing of 16S rRNA gene**

The amplification of the 16S rRNA *gene* was carried out by a PCR technique using the following primers: 5'-TCGAATTCGGATCCAGTITGATCCTGGCTC-OH-3' and 5'-TCGAAITCGGATCCAAGGAGGTGATCCAGCC-OH-3' according to Ausubel et al. (1999).

**Nucleotide sequence and the accession number**

The 16S rRNA gene of a pure culture of the most bioactive marine bacterial isolate generated in this study was sequenced and compared to the database presented at the GenBank.

**Effect of different culture media on antifungal production**

The antifungal agent production was carried out using five culture media, nutrient broth (NB) Oxide, king's medium B (Murray et al., 2003), a peptone water medium (PW) (Lab M Limited), it composed of (g/L): peptone (5.0), tryptone (5.0) NaCl (5.0). Two modified NB media were tested; the first composed of (g/L), NB, yeast extract (0.5), $NaNO_3$ (1.0), $K_2HPO_4$ (1.0), KCl (0.5), and $MgSO_4$ (0.5). While, the second modified medium was composed of (g/L); NB, $K_2HPO_4$ (11), $KH_2PO_4$ (5.5), $(NH_4)_2SO_4$ (1.2), $MgSO_4$ (0.4) and $CaCl_2$ (0.15). The pH of all these tested culture media was adjusted at 7.0. Erlenmeyer conical flasks containing 100 ml of each tested culture medium were inoculated with 1 ml (OD = 1.0) of pre-activated *B. pumilus* suspension. Then the flasks were incubated at 30°C for 48 h using a shaker incubator (150 rpm), aliquots of 5 ml of these cultures were taken, centrifuged at 12000 rpm for 20 min and used for measuring their bioactivity.

**Optimization of antifungal production using the Plackett-Burman experimental design**

Seven independent variables including three medium components; Tryptone, Peptone and NaCl, they were tested in three different concentrations 4, 5 and 6 g/l. In addition, four physiological conditions including; temperatures (25, 30 and 45°C), pH values (6, 7 and 8), inoculum sizes ($8.0 \times 10^6$, $7.3 \times 10^7$ and $9.8 \times 10^8$ cfu/ml) and incubation periods (24, 48, and 72 h) were also tested. These seven independent variables were screened in nine combinations organized according to the Plackett-Burman design matrix. For each variable, a high (+) and low (-) level was tested. All trials were performed in triplicates and the averages of observed activities were treated as the response. The main effect of each variable was determined using the following equation:

$$E_{xj} = (\Sigma M_{i+} - \Sigma M_{i-})/N$$

Where, $E_{xj}$ is the variable main effect, $\Sigma M_{i+}$ and $\Sigma M_{j-}$ are $\Sigma$ of suppression percentages of fungal growth in trials where the independent variable (xi) was present in high and low levels, respectively, and N is the number of trials divided by 2. Verification of validity of the optimum medium compared to the basal medium and the Plackett-Burman reverse medium was applied. The *t*-test statistical analysis was performed for equal unpaired samples to determine the variable significance (Bie et al., 2005).

**Application of a trickle flow column bioreactor for antifungal production**

In this part of work a trickle flow column bioreactor connected with a peristaltic pump was used to perform antifungal agent production. It was composed of a glass column (60 cm long, 5 cm internal diameter and a total workable volume of 500 ml), it was also connected at the top with an air pump having a flow rate of 1.0 L/min through sterilized air filters. The column was separately packed with 2 to 5 g of the tested solid supporters (sponge and Luffa pulp particles). The packed column and its parts were sterilized using an autoclave for 30 min. Then the sterilized packed column was inoculated with 100 to 150 ml of the antifungal producer suspension ($8.0 \times 10^6$ cfu/ml), and incubated at 25°C till a complete adsorption process of cells was observed. The optical density and pH were continuously detected in the effluent and the efficiency of the system was determined (El-Naggar et al., 2004).

**Scanning electron micrograph**

Scanning electron micrographs of the used solid supporters (sponge and luffa- pulp) with and without the adsorbed bacterial cells were captured at the Electron Microscopy Center, Faculty of Science; Alexandria University, Egypt.

**Purification of antifungal agent using thin layer chromatography**

***Preparation of the plates***

Glass plates (20 × 20 cm) were cleaned and air dried. About 40 g of silica gel G60 were suspended in 100ml of distilled water. The suspension was spread over the plates with a thickness of 0.1cm. The plates left for air dryness then activated by heating at 120°C for one hour. The $R_f$ of the active components was determined using 100ml of different solvent systems preformed in vol/vol as follows: benzene:chloroform, 50:50; benzene:acetone, 90:10; benzene: ethyl-format: formic acid, 75:24:1; toluene: ethyl-format: formic acid, 50:40:10; toluene: chloroform:acetone, 40:25:35; chloroform: methanol, 50:50; dichloromethane : methyl alcohol: water, 50:30:20; dichloromethane: methanol: water, 65:32:3.

The obtained spots were separately scratched and dissolved in dimethylsulfooxide. Then Silica gel was removed by centrifugation at 5000 rpm for 15 min and then the antifungal activity of each extract against the tested pathogenic fungi was estimated as

mentioned before.

**Bio-toxicity of the purified antifungal agent**

The toxicity bioassay was carried out according to Meyer et al. (1982) using 24 h old neuplii of *Artemia salina* as a biomarker. Different concentrations of the purified agent (100, 200, 1000, 1500, 2500 and 5000 ppm) were made and distributed separately in triplicate using clean and dry glass vials (20 ml) then completed to a total volume of 10 ml/each using sterile seawater. Ten live neuplii of *A. salina* were transferred to each vial. The number of the viable biomarker was counted after 24 h of application. The mortality percentages and the half lethal concentration ($LC_{50}$) were determined using the probit analysis method (Reish et al., 1987).

**A Comparison between the antifungal activity of the purified agent and some commercial antifungal drugs**

The bioactivity of 50 ppm the purified antifungal component in comparison to the bioactivity of 500 ppm/each of three commercial antifungal drugs based on the active fluconazole compound (Diflucan, Flocoral and Fungimycin) was estimated using *A. niger, F. solani, P. oxilacum, R. solani* and *C. albicans*. The suppression growth % of the pathogenic fungi was detected compared to the control (untreated fungi).

**Partial chemical characterization of the purified antifungal agent**

The UV/Visible spectrum of the most active spot was determined using spectrophotometer UV-vis./Jenway 6800 and the dimethyl-sulfooxide solvent as a blank. The infrared spectrum of the antifungal agent was carried out using a Peak Find-Memory-27 spectrophotometer at the Microanalysis Center, Cairo University, Egypt. The Mass spectrum of the antifungal agent was subjected using DI Analysis Shimadzu Qp-2010plus/mass spectrophotometer at the Microanalysis Center, Cairo University, Egypt.

## RESULTS

### Isolation and molecular identification of the most potent marine antifungal producer

A preliminary analysis for antifungal activity of different marine bacterial strains isolated from the eastern harbor and the western harbor of Alexandria, Egypt was conducted using some plant and human pathogenic fungi. All strains were grown on nutrient broth medium and then screened to select the most potent marine bacterial isolate acting against at least three pathogenic fungi with an activity >30%. It was showed that the marine bacterial isolate coded MS12 was the most potent isolate acting against the tested plant and human pathogenic fungi, *F. solani, R. solani* and *C. albicans*, the antifungal activities were 50, 43 and 32%, respectively.

The obtained amplified PCR fragment (835 bp) was purified and detected using agarose gel electrophoresis. Then the obtained amplified 16S rRNA was sequenced and compared with the data presented in the Genbank using Blast search program. It was found that the bacterial isolate MS12 had a new genomic sequence which indicate the isolation of a new strain of *Bacillus pumilus*, it genetically identified as *Bacillus pumilus*SMH101 with a new association No.KF964031.

### Effect of different culture media

Five different culture media were examined to obtain the highest antifungal activity from *B. pumilus*SMH101. It was found that the peptone water medium was the more effective medium tested compared to others. The percentage of the antifungal activity against *F. solani*, *R. solani* and *C. albicans* was 52.5, 47.1 and 34.9, respectively, (Table 1).

### Application of Plackett-Burman design

The components *of B. pumilus*SMH101 culture medium in addition to the physiological conditions were optimized for a maximum antifungal activity against *F. solani, R. solani* and *C. albicans*; it was carried out using the Plackett-Burman experimental design (Table 2).

The main effect of the tested variable was presented as the difference between the fungal suppression % averages at both the high level (+) and the low level (-) of the examined variable (Figure 1). The obtained data of the main effect as well as the *t*-test values showed the physiological conditions; inoculum size, temperature and the incubation period must be adjusted at their low levels ($8.0 \times 10^6$cfu/ml, 25°C and 24 h, respectively) to obtain more antifungal activities against *F. solani, R. solani* and *C. albicans*. Moreover, the inhibition of both *F. solani* and *R. solani* was maximized on the addition of the high level (6 g/L) of tryptone, while, the inhibition of *C. albicans* was maximized on adding the high level (6 g/L) of both peptone and NaCl.

### Verification of Plackett-Burman experiment

In order to validate the obtained results and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates to predict the near optimum levels of independent variables. The data were examined and compared to the basal and anti-optimized medium. It was revealed that the average antifungal activity against *F. solani, R. solani* and *C. albicans* by *B. pumilus*SMH101 was increased by 1.5, 1.5 and 1.9 fold, respectively, when grow on the optimized medium (data not shown). The results indicated for inhibiting both *F. solani* and *R. solani* the

**Table 1.** The effect of different culture media on the antifungal activity (%) of *B. pumilus*MSH101 using some plant and human pathogenic fungi as indicators.

| Culture Medium | [a]Antifungal activity (%) against | | |
|---|---|---|---|
| | ***F. solani*** | ***R. solani*** | ***C. albicans*** |
| Nutrient broth (NB) | 50 | 43 | 32 |
| King's medium | 38.0 | 29.8 | 18.0 |
| Peptone Water | 52.5 | 47.1 | 34.9 |
| Modified-NB-1 | 41.1 | 38.7 | 23.0 |
| Modified-NB-2 | 39.8 | 37.2 | 22.7 |

[a]Antifungal activity (%) = $\frac{\text{Control fungal diameter (mm) - Treated fungal diameter (mm)}}{\text{Control fungal diameter (mm)}} \times 100$

**Table 2.** The optimization of the antifungal production by *B. pumilus*SMH101 using the Plackett-Burman experimental design and its antifungal activities against some plant and human pathogenic fungi.

| Trial no. | Independent variables 1 | | | | | | | [a]Antifungal activity (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **I. P (Days)** | **I. S (ml)** | **Temp. (°C)** | **pH value** | **NaCl ($gl^{-1}$)** | **Pep. ($gL^{-1}$)** | **Try. ($gL^{-1}$)** | ***F. solani*** | ***R. solani*** | ***C. albicans*** |
| 1 | -1 | -1 | -1 | +1 | +1 | +1 | -1 | 2.8 | 1.8 | 2.1 |
| 2 | +1 | -1 | -1 | -1 | -1 | +1 | +1 | 64.3 | 58.6 | 36.2 |
| 3 | -1 | +1 | -1 | -1 | +1 | -1 | +1 | 44.7 | 42.1 | 23.8 |
| 4 | +1 | +1 | -1 | +1 | -1 | -1 | -1 | 54.3 | 50.6 | 27.5 |
| 5 | -1 | -1 | +1 | +1 | -1 | -1 | +1 | 52.9 | 50.4 | 33.5 |
| 6 | +1 | -1 | +1 | -1 | +1 | -1 | -1 | 34.2 | 29.4 | 22.8 |
| 7 | -1 | +1 | +1 | -1 | -1 | +1 | -1 | 44.5 | 42.3 | 19.0 |
| 8 | +1 | +1 | +1 | +1 | +11 | +1 | +1 | 2.9 | 2.4 | 2.3 |
| 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 52.5 | 47.2 | 34.9 |

[a]Antifungal activity (%) = $\frac{\text{Control fungal diameter (mm) - Treated fungal diameter (mm)} \times 100}{\text{Control fungal diameter (mm)}}$

culture should be formulated as follows (g/L): tryptone (6), peptone (5), NaCl (5), in addition, pH (7.0), inoculum size ($8.0 \times 10^6$ cfu/ml), temperature (25°C) and incubation period of 24 h. While, for inhibiting *C. albicans* the formula should be as follows: (g/l): tryptone (5), peptone (6), NaCl (6), in addition, pH (8.0), inoculum size ($8.0 \times 10^6$cfu/ml), temperature (25°C) and incubation period of 24 h.

## Application of trickle flow column

A trickle flow column bioreactor connected with a peristaltic pump was used to perform antifungal agent production. The column was separately packed of the tested solid supporters (sponge and luffa pulp particles). At the end of the incubation period, the antifungal activity was determined and compared to free cells of *B. pumilus*SMH101. The adsorbed cells on sponge particles led to antifungal activities of 50.9, 45.4 and 29.2% against *F. solani*, *R. solani* and *C. albicans*, respectively. The adsorbed cells on luffa pulp particles led to antifungal activities of 47.2, 42.2 and 23.8%, respectively, (Table 3). Moreover, the development of the bacterial biofilms on these used solid supporters was investigated using scanning electron microscopy (Figure 2).

## Bio-toxicity of the *B. pumilus*SMH101 purified extract using *A. salina* as a biomarker

The bio-toxicity of different concentrations (from 100 to 5000 ppm) of *B. pumilus*SMH101 purified extract were estimated using *A. salina* as a biomarker, then the mortality percent was calculated and presented using the probit analysis method. The results indicated the purified antifungal agent had a relatively low toxicity level (Table 4). The $LC_{50}$ (the concentration at which 50% of the tested biomarker individuals die) of this agent was 1072 ppm and it was estimated from the best fit line obtained on using the probit analysis method.

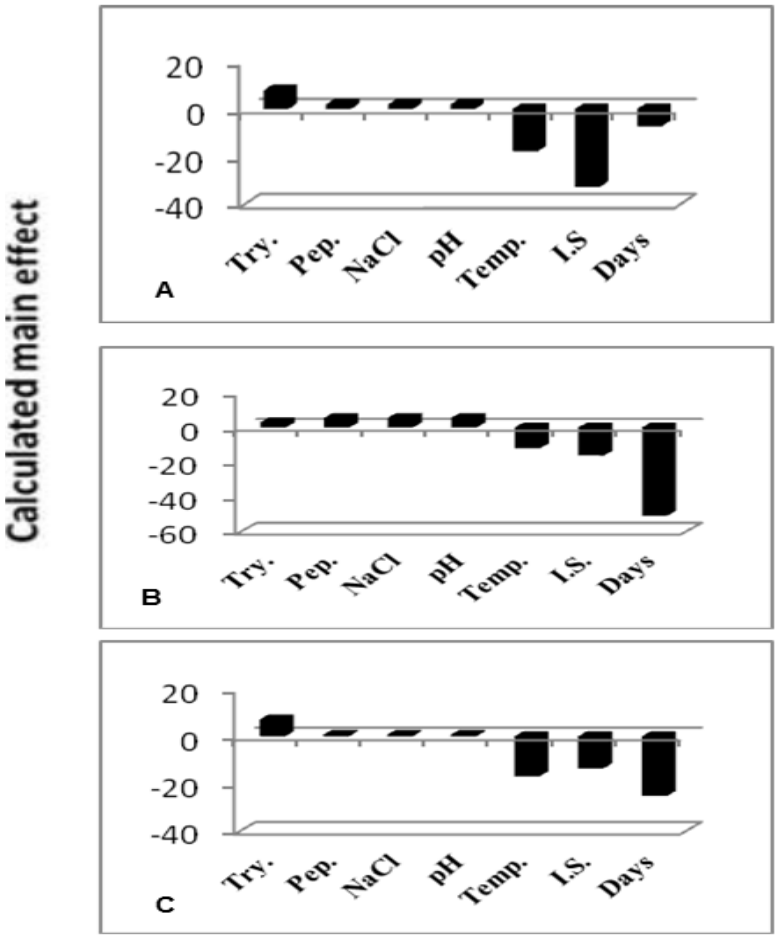


**Figure 1.** Elucidation of different cultured factors affecting the production of antifungal agent by *B. pumilus*SMH101 against *F. solani* (A), *C. albicans* (B) and *R. solani* (C), using the Placket Burman experimental design. Try. = tryptone, Pep = peptone, Temp. = temperature,

**A comparison of the antifungal activity of the partially purified agent of *B. pumilus*SMH101 to some commercial antifungal drugs**

The bioactivity of 50 ppm purified antifungal agent of the marine *B. pumilus*SMH101 was separately compared to 500 ppm of each commercial antifungal drug; this concentration is tenfold more than that of the produced antifungal agent of the marine *B. pumilus*SMH101. The data presented in Table 5 showed a broad antifungal spectrum of the purified agent of *B. pumilus*SMH101 with an average activity of 60% against the five tested human and plant pathogenic fungi compared to the tested commercial drugs based on the active fluconazole compound; Diflucan, Flocoral and Fungican; the obtained average inhibition percentage was 48, 49 and 54%, respectively. Moreover, the most inhibited fungus was *F. solani* (87%) followed by *R. solani* (80%) and *C. albicans* (70%) compared to the tested antifungal drugs; the average inhibition percent was 60, 55 and 70%, respectively. Moreover, the photographs in Figure (3) showed the inhibition percent obtained by the purified marine *B. pumilus*SMH101 agent (Figure 3-B) compared to the untreated fungi (control) (Figure 3-A).

**Partial Characterization of the *B. pumilus*SMH101 purified antifungal agent**

The chemical characterization presented in Figure (4) showed the UV-Vis, infra-red (IR), and Mass spectra of the purified antifungal agent. The UV spectrum of the compound (Figure 4-A) resulted in a single peak appeared at $\lambda_{280}$nm which proved the aromatic character of the compound. The IR spectrum showed seven absorption bands (Figure 4-B) the major five bands were

**Table 3.** Antifungal activity (%) of the adsorbed *B. pumilus*SMH101 cells against *F. solani*, *R. solani* and *C. albicans* using a glass trickle flow column and the optimized culture medium.

| Supporter Packing material | [a]Antifungal activity (%) against | | | Average |
|---|---|---|---|---|
| | *F. solani* | *R. solani* | *C. albicans* | |
| Control (Free cells) | 69.2 | 62.5 | 62.5 | 58.6 |
| Sponge | 50.9 | 45.4 | 29.2 | 41.8 |
| Lufa pulp | 47.2 | 42.3 | 23.8 | 37.8 |

[a]Antifungal activity (%) = Control fungal diameter (mm) - treated fungal diameter (mm) × 100
Control fungal diameter (mm).

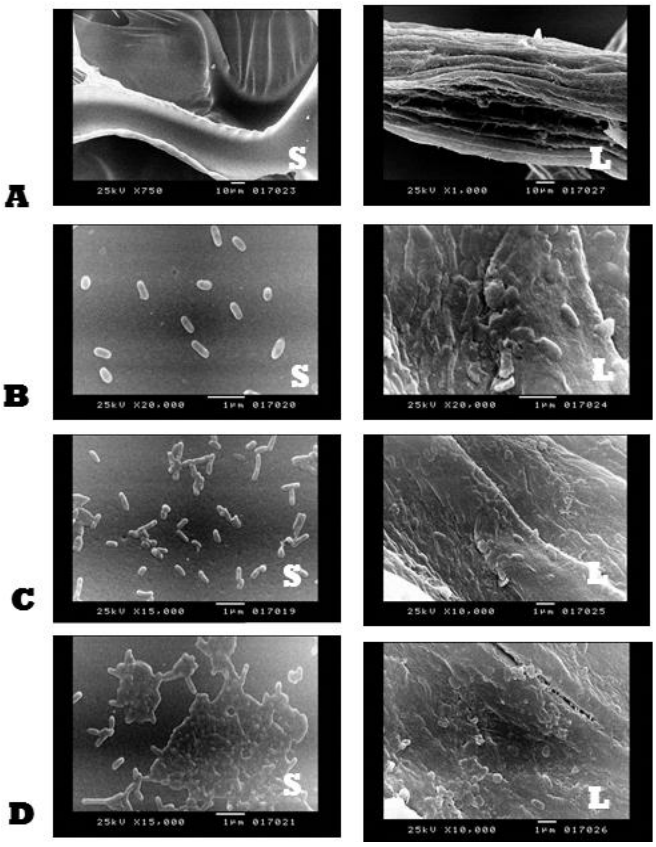


**Figure 2.** Micrographs show the development of the adsorbed B. pumilusSMH101 biofilm on sponge (S) and luffa pulp (L) as packing supporters of the used glass trickle flow column after 2 days (B), 4 days (C), and 6 days (D) compared to the un-inoculated supporters (control) (A).

explained as follows; the first band appeared at 3441 cm which indicated the presence of $NH_2$, OH or NH groups, the second band appeared at 2966.95 cm which indicated the presence of the aromatic C-H group, the third band appeared at 1638.23 cm which indicated the at 1457.92 cm which indicated the presence of aromatic ring in the compound.

The last band appeared at 1051.01 cm which indicated the presence of the ether linkage in this compound. Moreover, the obtained Mass spectrum of this compound (Figure 4-C) showed the appearance of a molecular ion peak at m/e = 888.40, while, the base ion peak was appeared at m/e =132.10. The molecular weight of this compound showed to be 875Da.

**Table 4.** The bio-toxicity of different concentrations of the purified antifungal agent of B. pumilusSMH101 using Artemia salina as a biomarker.

| [a]concentration (ppm) | Log concentration | Mortality % after 24 h |
|---|---|---|
| 100 | 2.00 | 10 |
| 500 | 2.7 | 18 |
| 1000 | 3.00 | 27 |
| 1500 | 3.18 | 57 |
| 2500 | 3.40 | 80 |
| 5000 | 3.70 | 97 |

[a]The value of LC50 was detected using the probit analysis, it was 1072 ppm.

## DISCUSSION

Genus *Bacillus* has a long and distinguished history in the field of biotechnology. Since, members of this genus are used for the synthesis of a very wide range of important medical, agriculture, pharmaceutical and other industrial products including antibiotics, bacteriocins, enzymes, amino acids, sugars, surfactants and flavor enhancers (Anthony et al., 2009; Bhaskar et al., 2007; Lisboa et al., 2006; Parvathi et al., 2009; Ying et al., 2005).

**Table 5.** The antifungal activity (%) of the partially purified agent of *B. pumilus*SMH101 (0.05 mg/ml) compared to the antifungal activity (%) of some commercial antifungal drugs based on the active fluconazole compound (0.5 mg/ml) using different plant and human pathogenic fungi.

| Types of antifungal agent | *Antifungal activity (%) | | | | | |
|---|---|---|---|---|---|---|
| | *F. solani* | *R. solani* | *C. albicans* | *A. niger* | *P. oxalaticum* | 61 |
| *B. pumilus* agent | 87 | 80 | 70 | 23 | 44 | 48 |
| Diflucan | 55 | 51 | 78 | 26 | 28 | 49 |
| Flucoral | 57 | 52 | 80 | 27 | 29 | 51.8 |
| Fungican | 70 | 64 | 65 | 25 | 35 | |

*Antifungal activity (%) = $\frac{\text{Control fungal diameter (mm) - treated fungal diameter (mm)}}{\text{Control fungal diameter (mm)}} \times 100$

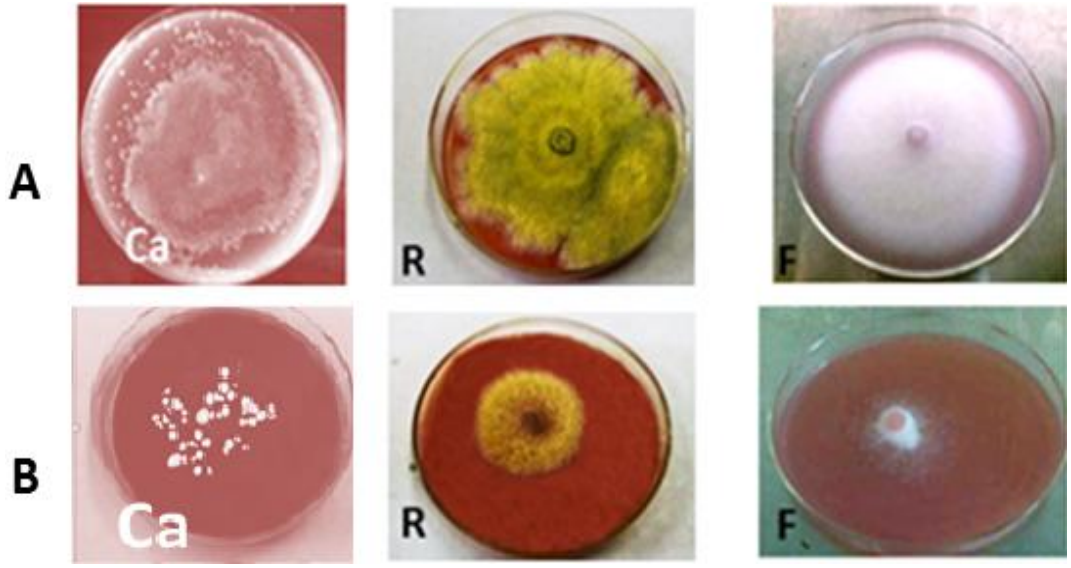


**Figure 3.** Photographs show the antifungal activities of the purified marine *B. pumilus*SMH101 agent (B) against *C. albicans* (Ca), *R. solani* (R) and *F. solani* (F) compared to the untreated fungi (control) (A).

Statistical experimental designs are powerful tools for an economic and a rapid search of the key factors from a multivariable system. It minimizes the error in determining the effect of these variables on the growth of the used microorganisms (Abou-Elela et al*.,* 2009; Xiong et al*.,* 2007). So, Plackett-Burman experimental design was used to optimize the components of the culture medium and reflect the relative importance of various environmental factors on the production of the antifungal agent by *B. pumilus*SMH101 in liquid cultures. It was found the optimum physiological conditions for antifungal agent production by *B. pumilus*SMH101 were achieved on applying the tested low levels; the temperature was 25°C, the inoculum size was $8.0 \times 10^6$ cfu/ml and the incubation period was 24 h, regardless the target pathogen. While the optimum culture components showed insignificant effect on the production of the antifungal agent by *B. pumilus*SMH101 and they varied according to the pathogenic target (Figure 1 and Table 2).

Cell immobilization shows many operational and economic advantages such as prolong metabolic activities, reuse of the biocatalyst, increase of cell concentration in preventing washing out of cells at high flow rates (Gautam et al*.,* 2002). However, the results obtained on using immobilized *B. pumilus*SMH101 cells (absorbed bacterial cells on solid supporters; luffa pulp and sponge) showed low antifungal activities against *F. solani*, *R. solani* and *C. albicans*. The average activity of the adsorbed cells was 37.8 and 41.8%, respectively, compared with the free bacterial cells of *B. pumilus*SMH101 58.6% (Table 3). Similar results were obtained by Freeman and Aharonowitz (1981) who developed a mild new method for the immobilization of the whole microbial cells. They found the yield of cepha losporins antibiotic

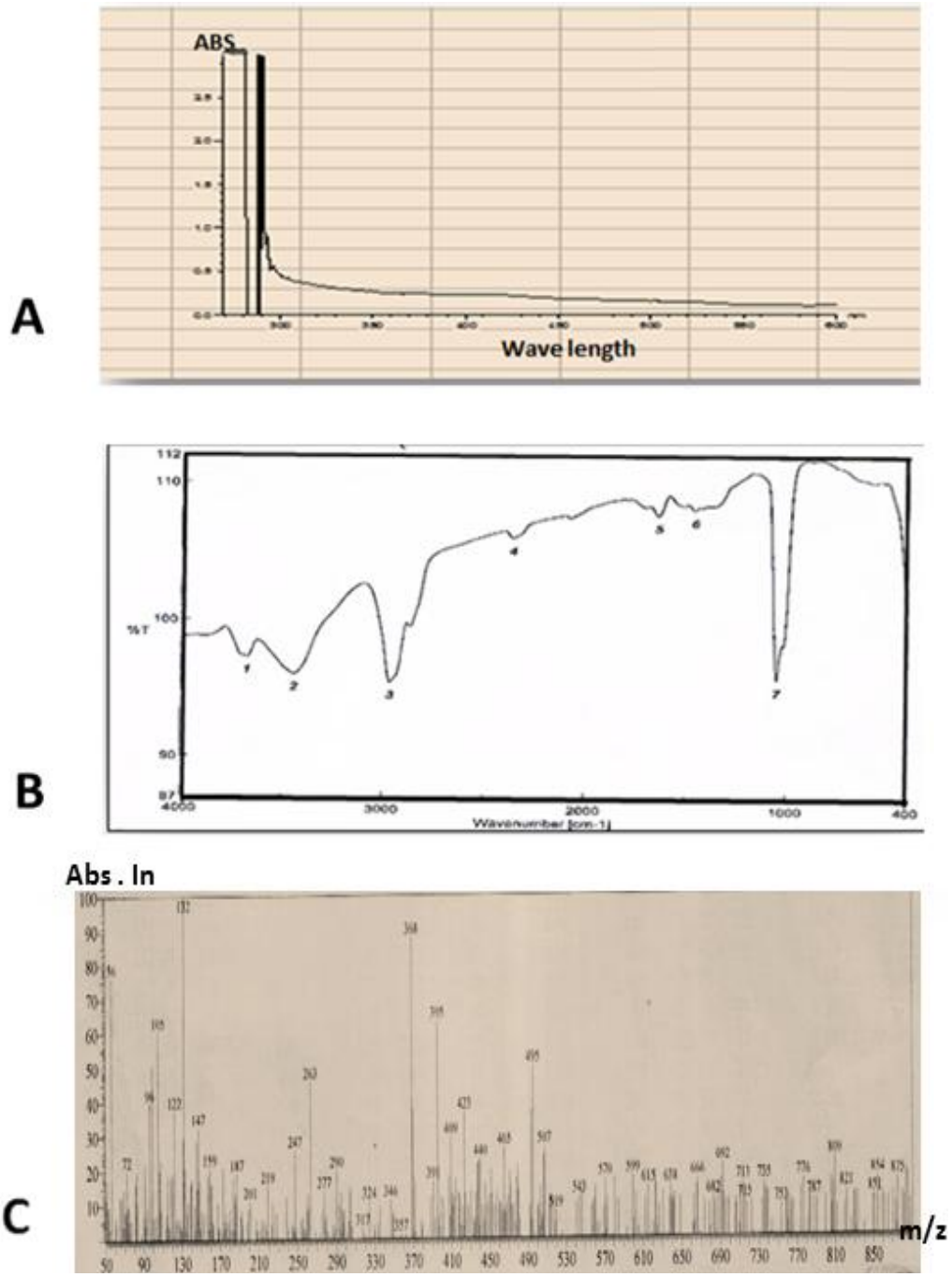


**Figure 4.** Some chemical characterization of the antifungal active component produced by *B. pumilus*SMH101 using dimethylsulfooxide (DMSO) as a blank. UV/Vis. Spectrum (A), IR spectrum (B) and Ms Spectrum (C).

production by the immobilized bac-terial cells using acrylamide monomers (direct poly-merization method) was significantly decreased com-pared to the free cells of *Streptomyces clavuligerus.* Moreover, Punita et al. (2007) used Immobilized *Acremonium chrysogenum* (mold) cells for cephalo-sporin-C production. It was found that cell growth rate of immobilized cells was reduced with about 39% of the growth rate of free cells. Contrary, Srinivasulu et al. (2003) studied the immobilization effect of *Streptomyces marinensis*NUV-5 using calcium alginate

for the production of neomycin. They reported the antibiotic productivity was enhanced with 32% on the use of the immobilized cells over the use of the conventional free-cell.

The fungicidal activity of another *B. pumilus* strain was investigated by Bottone and Peluso (2003). They found that a compound produced by *B. pumilus* inhibits *Mucor* and *Aspergillus* species through the inhibition of spore germination and aborted elongating hyphae. The molecular mass of this compound was determined by diffusion through dialysis membrane to be 500 to 3000 Da. These findings were agreed with the results obtained in this study; the photographs of the purified antifungal agent indicated the fungicidal action against *F. solani* and *R. solani* was carried out through inhibiting the hyphae elongation (Figure 3), the bioactivity was 87 and 80%, respectively, compared to the control (Table 5). Also, the determined molecular weight (875 Da) of this antifungal agent through MS-spectrometry was located between 500 and 3000 Da. Also, Aunpad and Na-Bangchang (2007) isolated *B. pumilus*WAPB4, it showed a remarkable antibacterial activity against methicillin-resistant *Staphylococcus aureus*MRSA, vancomycin-resistant *Enterococcus faecalis*VRE, and several Gram-positive bacteria. This Bacteriocin was designated as pumilicin-4 with a molecular mass of 1994.62 Da using a mass spectrometry.

Moreover, the obtained *B. pumilus*SMH101 antifungal agent found to have an antagonistic action against the tested *C. albicans* with a bioactivity of 70% compared to the control (Figure 3). Contrarily, Guo et al. (2009) used thymol (THY) which was found to have *in vitro* antifungal activity against 24 fluconazole (FLC)-resistant and 12 FLC-susceptible clinical isolates of *C. albicans* but no antagonistic action was observed.

In general, the results of the bio-toxicity test of the obtained purified antifungal agent of *B. pumilus*SMH101 showed a relatively low toxicity level where the $LC_{50}$ was 1072 ppm which is much more than the concentration used in the application process of this study (Table 4). Dissimilarly, many authors worked on using *B. pumilus* in order to obtain bioactive secondary metabolites acting against different pathogenic fungi regardless the biotoxicity of these metabolites towards human or plant (Ghasemi et al., 2012; Munimbazi and Bullerman, 1997, 1998; Munimbazi and Bullerman, 1998). On the other hand, Yadav et al. (2007) purified a cytosolic protein from *E. coli* BL21; it demonstrated potent antifungal activity against pathogenic strains of *Aspergillus* species (*A. fumigatus, A. flavus, A. niger*) and *C. albicans* with MIC of 1.95 to 3.98 and 15.62 mg $ml^{-1}$, respectively, and it showed no cytotoxicity up to 1250 mg $ml^{-1}$ *in vitro* toxicity tests, which is very high concentration compared to the $LC_{50}$ of the antifungal agent of *B. pumilus*SMH101 (Table 4).

Finally, from the results of the comparative study it can concluded this produced safe antifungal agent from the marine *B. pumilus*SMH101 may act as a promising alternative tool for the treatment of some pathogenic fungi; *F. solani*, *R. solani* and *C. albicans* even on using a very low concentration (0.05 mg/ml) (Table 5), this is to face the increasing in the pathogenic fungus resistance towards the used commercial antifungal drugs especially those based on the active fluconazole compound. On the other hand, many investigations will be carried out in order to precisely identify the main active component in this produced antifungal agent.

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