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Full Length Research Paper

Identification and Improving Antibiotic Production by Some Bacteria Isolated From Egyptian Soil

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The three isolates (SOF12, AMF11 and SMF4) showed highly capability for antimicrobial activity against all the tested pathogenic bacteria with high potential inhibitory activity against yeast and two filamentous plant pathogenic fungi were prescreening studies for bioassay against crude extracts and assayed for their minimal inhibitory concentrations (MIC). The result showed that, crude extract tested positive in bioassay against the all tested pathogenic bacteria while different between them. MIC values ranging from 0.0625 to 5 mg/ml. Based on it's morphological and microscopy characteristics as well as 16S rRNA sequence analysis, the three isolates designated as SOF12, AMF11 and SMF4 were identified as identified *Bacillus megaterium* TDB-13, *Bacillus licheniformis* DV7 and *Bacillus subtilis* 003141, respectively. To study the effect of some growth factors on the production of antagonistic bacteria metabolites against selected human, plant pathogenic bacteria and plant pathogenic fungi and yeast. The results showed that the antimicrobial activity of the metabolites increased generally from the first to the 9 day of incubation, the optimum temperature for maximum activity by *B. megaterium* and *B. licheniformis* was observed at 30°C while *B. Subtilis* at 25°C, the optimum pH for *B. megaterium* and *B. licheniformis* was 7 whereas *B. subtilis* at pH 8, the best carbon sources for *B. megaterium* was starch and asparagine, while *B. licheniformis*, starch and glycerol, but *B. subtilis* glycerol. The asparagine was observed as the best nitrogen source for all the strains.

Keywords: Antimicrobial agent, Bacterial isolates, growth factors, 16S rRNA gene.

INTRODUCTION

Antagonistic bacteria produce antimicrobial substances as important compound for self-defense function towards other organisms e.g., *Bacillus* sp. producing antimicrobial compounds have been used as biocontrol agents against plant pathogenic fungi (Yilmaz *et al.*, 2005). Similarly,

Burkholderia cepacia complex formerly known as *Pseudomonas cepacia* is a group of nine closely related bacterial species that have useful properties in the natural environment (Chiarini *et al.*, 2006) have emerged as powerful biocontrols agents for plant pathogens (Bevivino *et al.*, 1998). However, the production of antimicrobial substances depends upon the substrate medium for their optimal growth, temperature, pH and the concentration of nutrients in the medium (Leifert *et al.*, 1995). Many studies

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exploring of beneficial organisms have been carried out, such as *P. fluorescens*, which was one of the examples used for the control of *Fusarium* wilt of tomato. Similarly, *P. fluorescens* were found to be effective biocontrol agents against the Phytophthora disease in black pepper (Diby *et al.*, 2005). In addition, most of the species from the genus *Bacillus* are considered as safe microorganisms and they possess remarkable abilities to synthesize many substances that have been successfully used in agriculture and for industrial purposes. The secondary metabolites produced by several species and strains of the genus *Bacillus* have been found to show antibacterial or antifungal activity against different phytopathogens (Ongena and Jacques 2008). Carbon as a part of an ingredient in the medium is required for bacterial growth and to enhance the production of antimicrobial substances. Antimicrobial substances produced by bacterial species were greatly influenced by variation of carbon sources (El-Banna *et al.*, 2006). Galactose and glucose strongly enhanced the antimicrobial activity of *Corynebacterium kutscheri* and *C. xerosis* respectively, while ribose and lactose repressed their activity (Gebreel *et al.*, 2008). *P. fluorescens* in the liquid medium with the addition of glucose and sucrose produced more antimicrobial substances like 2, 4-diacetylphloroglucinol (Duffy and Defago, 1999). Balanced ingredient in medium as nutrition for bacterial growth and production of antimicrobial substances is important.

From the previously studies (Eman *et al.*, 2015) the three isolates (SOF12, AMF11 and SMF4) showed highly capability for antimicrobial activity against all the tested pathogenic bacteria (*Agrobacterium tumefaciens*, *Erwinia carotovora*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), with high potential inhibitory activity against yeast (*Candida albicans* and *Candida neoformans*) and two filamentous fungi pathogenic plant (*Fusarium oxysporum* and *Fusarium solani*). These bacterial were selected based on the inhibition zone over 25 mm and hyphal growth inhibition over 80% for determination of crude extracts and assayed for their minimal inhibitory concentrations (MIC). Moreover, to identify the potential antagonistic bacteria strain isolated as well as to study the effect of some growth factors on the production of antagonistic bacteria metabolites against selected human, plant pathogenic bacteria and plant pathogenic fungi and yeast.

Hence, the objectives of this study was to identify the potential antagonistic bacterial strain isolated from different soil types in Egypt (Eman *et al.*, 2015), and to study the effect of some growth factors on the production of antagonistic bacteria metabolites against selected human and plant pathogenic bacteria as well as plant pathogenic fungi and yeast.

MATERIALS AND METHODS

Preparation of crude extract

The crude antibiotic extract was prepared by extracting with organic solvents. Five millilitres (5 ml) of the isolate was inoculated into five (500 ml) flasks containing 300 ml Luria Bertani (LB) liquid medium (Eman *et al.*, 2015). The flasks were incubated at 37°C for 10 days and then centrifuged at 15000 rpm for one hour. The supernatant (about 1350 ml) was filtered and transferred into a clean one litre separating funnel in 500 ml quantities. This was extracted five times with 50 ml portions of petroleum ether which were pooled together and evaporated to dryness. The extraction was continued several times with 50 ml portions of ethyl acetate. The ethyl acetate extracts were also pooled together and evaporated to dryness. The ethyl acetate extract was then used as a crude sample for bioactivity assay.

Screening for antimicrobial activities

Microorganisms used in this study (Tested organisms)

The following test pathogenic organisms were used as indicator microorganism in all assays for determination of the screening isolates and metabolites produced (antibiotic). The test organisms, namely, the bacteria Gram negative [*Agrobacterium tumefaciens* (plant pathogen), *Erwinia carotovora* (plant pathogen), *Escherichia coli* (human pathogen), *Pseudomonas aeruginosa* (human pathogen)] and Gram positive [*Staphylococcus aureus* (human pathogen), Methicillin-resistant *Staphylococcus aureus* (MRSA, human pathogen) and the fungi [*Fusarium oxysporum* (plant pathogen), *Fusarium solani* (plant pathogen)], *Candida albicans* and *Candida neoformans* were all from the culture collection in the Department of Agricultural Microbiology, National Research Centre.

Preparation of tested bacteria

All tested bacteria were grown in LB broth medium and incubated at 37 °C for 24 hr and a total of 10 ml of the pure culture was centrifuged to pellet out the cells, washed twice with sterile physiological saline and the suspension adjusted to optical density 0.1 at 600nm which is equivalent to a cell population of about 10⁶ cells/ml on the McFarland standard. Bacteria suspension was stored in test tubes and refrigerated at 4 °C. These test bacteria were frequently checked for viability and prepared when appropriate.

Preparation of test fungi

All test fungi were grown on Potato dextrose agar (PDA)

plate for 48 hr and then re-suspended in Nutrient broth. *Candida albicans* was standardized to McFarland standard of 0.1 at OD 600nm before use. Fungal spores (*F. solani* or *F. oxysporum*) were harvested from 2-weeks old PDA cultures. An amount of 5 ml of sterile water, containing 0.05% (v/v) tween 80 (Sigma, St. Louis, MO) to improve the wetting properties of the solution, was added to a Petri plate culture, the spores were gently dislodged from the surface with a sterile glass rod, and suspensions were filtered through three layers of cheesecloth to remove mycelial fragments. Spore concentration was adjusted using a hemocytometer to obtain 10^6 conidia m^{-1} (Cantwell, 1970; Paul, 1975; Strober, 1997).

Bioassay of antimicrobial metabolite of isolates SOF12, AMF11 and SMF4

Bacterial and yeasts

The crude ethyl acetate extracts was screened for antibacterial activity using the agar disc diffusion method. In this method Luria-Bertani (LB) agar plates were prepared, sterilized and solidified (Eckwall and Schottel, 1997). After solidification pathogenic microorganisms cultures were swabbed on these plates. The sterile disc was dipped in the $500\mu\text{l ml}^{-1}$ of different crude ethyl acetate extracts and then placed on the surface of an agar plate that has been seeded with the organism to be tested and kept for incubation at 37°C for 24 hrs. Zone of inhibition was measured and recorded. The ratios of clear zone to colony were calculated by dividing the area of the clear zone by the area of the colony. Zone of inhibition was measured and compared with control disc negative control treatment with cell-free water extract. The experiments were repeated thrice and mean values of zone diameters were presented.

Hyphal inhibition by bacteria

Each crude ethyl acetate extracts was streaked onto PDA plate and a mycelia plug from an actively growing fungal colony (*F. solani* or *F. oxysporum*) was placed about 0.5 cm from the edge of crude ethyl acetate extracts streak and incubated at room temperature for 7 days. Fungal inhibition was observed every day (Jimenez-Esquelin and Roane, 2005). The radii of the fungal colony on the test and control plates were then measured. The percentage of hyphal growth inhibition was calculated using the following formula:

$$\% \text{ inhibition} = 100 - \left(\frac{R^2}{r^2} \times 100 \right) \text{ (Gamliel et al., 1989)}$$

R = radius of treated colony, r = radius of the control colony

Minimal inhibitory concentrations (MIC)

Approximately 1mg of the dried crude extract was weighted

and dissolved in 5 μl of 100% methanol and 95 μl of LB Broth at pH7.0. The extract was diluted to concentration of 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625mg/ml, each tested in triplicate, respectively.

For the bacteria and yeast, the extracts were incorporated into LB or PDB broth medium and inoculated with pure strains of Gram negative, Gram positive bacteria and yeast. A control tube containing the growth medium and the bacteria was set-up. The mixtures were incubated at appropriate temperature of 37°C for 24h in an orbital rotary shaker at 120 rpm. The minimum inhibitory concentration (MIC) of the extracts was regarded as the lowest concentration of the extract that did not permit and turbidity or growth of the test organisms. (Zahran et al., 2015).

For the fungi, the MIC were measured by incubation into 100ml PDB liquid media supplemented with sterile disc, dipped in each extract diluted concentration (0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625mg/ml). The aliquot (0.5 ml) of spore suspension (prepared by overtaxing a 7 mm disc, from the margin 7 days old colony in 10 ml sterile distilled water) were used as inoculum and incubated for 7 days in an orbital rotary shaker at 120 rpm and 27 °C. Mycelia were harvested, washed twice with distilled water and dried at 60°C for 3 days using aluminum foil cups for determination of mycelia dry weight. The percentage of hyphal growth inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Dry weight of control} - \text{Dry weight of treatment}}{\text{Dry weight of control}} \times 100$$

Identification of selected bacterial isolates

The most efficient antibiotic producing bacterial isolates were subjected to a set of morphological, physiological, biochemical tests as well as 16S rRNA gene sequencing for the purpose of identification.

Morphological characterizations

Macroscopic characterization

The isolates were grown on the surface of Luria-Bertani (LB) agar plates and their morphological features such as margins, pigmentation, colour and texture were observed.

Microscopic characterizations

The microscopic morphological features of the isolates were analyzed by using a Zeiss light microscope (Zeiss, West Germany, Germany). Gram stain reaction and motility tests were conducted. All reagents used were obtained from Fisher Scientific (Leicestershire, UK). The

gram-positive isolate was subjected to the modified Moeller's spore staining.

Biochemical tests

Biochemical tests were carried out according to the method of Cappuccino and Sherman (1992) with 24 hr old cultures.

Molecular characterization based on 16S rRNA gene

Genomic DNA was extracted from strains using the Wizard Genomic DNA kit (Promega, Madison, WI, USA) using the protocol according to the manufacturer instructions. 16S rRNA genes were amplified using universal eubacterial primers 8F (5'-AGAGTTTGATCMTGGCTCAG) and 1492R (5'-GGYTACCTTGTTACGACTT-3') (Lane *et al.*, 1985). 25 µL PCR reactions consisted of PuReTaq Ready-to-go PCR beads (GE Healthcare Buckinghamshire, UK), 0.5 µL of template genomic DNA, 400 nM of each primer. The thermocycler program consisted of 95°C for 10 minutes followed by 40 cycles of: 95°C for 30 seconds, 48°C for 30 seconds and 72°C for 1 minute 30 seconds. The program ended with a final extension step of 72°C for 10 minutes.

PCR products were column-purified using QIAquick PCR Purification Kit (Qiagen Valencia, CA, USA). They were sequenced using the universal primer 519F (5'-CAGCAGCCGCGGTAATAC) on an ABI 3730 DNA Analyzer at the Molecular Biology Resource Facility at the University of Tennessee, Knoxville, TN, USA.

High quality sequences (~950 bp) imported into the DNASTAR Lasergene v.7 software package, aligned using Mega Align, and a phylogenetic tree was constructed using ClustalW (Thompson *et al.*, 2003) program contained within Lasergene. Pairwise distances between sequences (used for phylogenetic tree construction) were also exported in tabular format.

Effect of some growth factors on antimicrobial metabolite production

Certain parameters of growth such as period of incubation (from 1 to 12 days), temperature (from 25 to 45 °C), pH (from 4 to 9) of the fermentation medium as well as effects of various sources of carbon (glucose, galactose, xylose, sucrose, mannitol, lactose, starch, fructose and maltose) and nitrogen (sodium nitrate, potassium nitrate, ammonium chloride, ammonium nitrate, asparagine, ammonium phosphate and ammonium sulphate) were evaluated for optimum production of antimicrobial metabolites.

Incubation periods

The isolates (1 ml each) were inoculated into three tubes of 10 ml sterile Luria-Bertani (LB) broth and incubated at

37°C. During the incubation period, samples were taken every 24 hours for twelve days, centrifuged to remove the cells and the supernatant liquid analyzed for inhibitory activity as in described in previously. This was done in duplicates and the results recorded.

Temperature and pH

The isolates (1 ml each) were inoculated into five of 10 ml Luria-Bertani (LB) broths and incubated at different temperatures (25, 30, 35, 40 and 45°C), varying pH values (4, 5, 6, 7, 8 and 9) for 72 hours. The cultures were then centrifuged and metabolite solutions obtained were then analysed for antimicrobial activity. The experiment was performed in duplicates and the results recorded.

Carbon and nitrogen sources

The effect of carbon and nitrogen sources on antimicrobial metabolite production was verified by fortifying the production medium with different sources of carbon and nitrogen. Sixty milligrams (60mg) of the various carbon and nitrogen compounds were separately added to 10 ml quantities of the fermentation medium in test tubes and sterilized. One millilitre of an isolate suspension was inoculated into each of the media aseptically and incubated at 37°C for 72 hours. The cultures were then centrifuged and the metabolite solutions obtained were tested for antimicrobial activity. The experiment was replicated for each of the isolates and the results recorded.

Statistical analysis

All the experiments were carried out in five replicates and mean values were presented. The data presented in graphs and tables corresponding to mean values + SEM and the statistical significant ($P < 0.05$) was established by using Graph Pad prism 5 software

RESULTS

Extraction and bioassay of antimicrobial metabolite of isolates SOF12, AMF11 and SMF4

The ethyl acetate extract appeared as a dark brown precipitate with a yield of 0.0137%w/v. Ethyl acetate crude extracts at a concentration of 200 µg ml⁻¹ were primarily tested for antimicrobial activity against the tested microorganisms by the disc diffusion method. The result showed that, crude extract tested positive in bioassay against the all tested pathogenic bacteria while different between them. According to the ethyl acetate crude extract from isolate SOF12 showed the highest active extracts followed by AMF11 and SMF4 **Table 1**. From all crude

Table 1: Antimicrobial activity of each type of crude extracts against test microorganisms

Crude extract	Test microorganisms									
	Bacteria						Yeast		Filamentous Fungi	
	Human pathogenic bacteria				plant pathogenic bacteria					
	SA	MRSA	EC	PA	AT	EC	CN	CA	FS	FO
SOF12	31.0	29.6	17.3	17.5	25.4	16.9	21.0	15.7	75%	82%
AMF11	28.3	33.0	31.5	22.0	23.7	21.3	32.0	24.2	67%	62%
SMF4	19.0	24.6	11.0	15.2	24.9	15.2	21.5	12.4	62%	51%

Table 2: Minimum inhibitory concentrations ($\mu\text{g/ml}$) of the selected antimicrobial active isolates against the test organisms

Crude extract	Test organisms									
	Human pathogenic bacteria				Human pathogenic bacteria		Yeast		Filamentous Fungi	
	Gram positive		Gram negative							
	SA	MRSA	EC	PA	AT	EC	CN	CA	FS	FO
SOF12	0.0625	0.125	0.125	0.0625	0.0625	0.125	0.25	0.125	0.125	0.0625
AMF11	0.125	0.25	0.125	0.125	0.25	0.125	0.25	0.25	0.5	0.25
SMF4	0.125	0.125	0.0625	0.25	0.25	0.0625	0.50	0.25	0.125	0.25

extract, the extract of SOF12 were highly against *S. aureus* (31.0 mm), MARS (29.6 mm), followed by *A. tumefaciens* (25.4 mm) and the least *P. aeruginosa* (17.5 mm), *E. coli* (17.3 mm) and *E. carotovora* (16.9 mm). While at yeast, the highly active extracts were against *C. Neoformans* (21.0 mm) and the least *C. albicans* (15.7 mm). The highly hyphal growth inhibition of active extracts were against *F. oxysporum* (82%) followed by *F. solani* (75%). While the extract of AMF11 were exhibited the most activity against MRSA (33.0 mm), *E. coli* (31.5 mm), *S. aureus* (28.3 mm), followed by *A. tumefaciens* (23.7 mm), *P. aeruginosa* (22.0 mm) and the least *E. carotovora* (21.3 mm). While at yeast, the highly active extracts were against *C. neoformans* (32.0 mm) and the least *C. albicans* (24.2 mm). The active extracts were against *F. solani* (67%) followed by *F. oxysporum* (62%). On the other hand, SMF4 showed less and smaller zone of inhibition compared to the other two bacterial strains (SOF12 and AMF11).

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) values ranging from 0.0625 mg/ml to 5 mg/ml.

Isolate SOF12 showed highest activity against *S. aureus* and *Ps. Aeruginosa* with an MIC of 0.0625 $\mu\text{g/ml}$ whereas the MIC for MRSA and *E. coli* was 0.125 $\mu\text{g/ml}$ (Table 2). Isolate AMF11 showed good antimicrobial activity against all the test organisms used especially the *S. aureus*, *E. coli*

and *Ps. aeruginosa*, with MICs of 0.125 $\mu\text{g/ml}$ but least activity against MRSA with MIC of 0.25 (Table 2). Isolates SMF4 was also strongly inhibitory with values of 0.0625 $\mu\text{g/ml}$ against *E. coli* while least activity against *Ps. aeruginosa* with MIC of 0.25. It appears that the extracts are more active against Gram negative bacteria than Gram positive ones.

For yeast and filamentous fungi, isolate SOF12 showed highest activity against *F. oxysporum* with an MIC of 0.0625 $\mu\text{g/ml}$, while isolate AMF11 leas activity *F. solani* with an MIC of 0.5. The isolate SMF4 was moderate activity against all the yeast and filamentous fungi tasted.

Identification of selected bacterial isolates

The three isolates showed different capability in antimicrobial activity against pathogenic microorganisms, SOF12, AMF11 and SMF4 were identification. From the morphology and biochemical reaction of the three isolates SOF12, AMF11 and SMF4 was found to be a Gram positive rod bacterium displaying a number of characteristics matching the *Bacillus* species listed in the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Molecular characterization based on 16S rRNA gene

The three selected bacterial strains were further identified on the basis of sequence analysis of 16S rRNA. Based on

Table 3: Percent identity of bacterial isolates with other known identified bacterial strains

Genes	Strain	Similar strain	Similarity (%)	Accession No.
16S genes	SOF12	<i>Bacillus megaterium</i>	95%	TDB-13
	AMF11	<i>Bacillus licheniformis</i>	95%	DV7
	SMF4	<i>Bacillus subtilis</i>	90%	003141

the phylogenetic tree and the data in tabular format which showed the percent identity (Table 3), strains SOF12 and AMF11 had 95% homology in 16S rRNA sequence to *Bacillus megaterium* TDB-13 (SOF12) and *Bacillus licheniformis* DV7 (AMF11), respectively whereas strain SMF4 had 90% homology to *Bacillus subtilis* 003141 (Fig. 1).

Effect of some optimization of fermentation parameters growth on antimicrobial metabolite production

Process optimization was carried out including incubation period, initial pH, temperature, carbon source and nitrogen source must be optimized in order to achieve higher yields of antimicrobial metabolite. Optimization process was carried out varying one parameter at a time. All experiments were conducted in three replicates and the mean values are presented.

Effect of incubation period

The antimicrobial activity against *A. tumefaciens*, MRSA and *F. oxysporium* by *B. megaterium*, *B. licheniformis* and *B. subtilis* when the LB and PDB medium was incubated under different incubation periods (from zero to 12 days) is presented in the following Figures (2). The fermentation was carried out at 30 °C and other experimental conditions were kept constant. The optimum period achieved was taken for further experiments. The antimicrobial activity of the metabolites increased generally from the first to the 9 day of incubation, after which the activity became stable (Figures 2). This was the case for all the strains (*B. megaterium*, *B. licheniformis* and *Bacillus subtilis*).

Effect of temperature on antibiotic production

The antimicrobial activity against *A. tumefaciens*, MRSA and *F. oxysporium* by *B. megaterium*, *B. licheniformis* and *B. subtilis* when the LB and PDB medium was incubated at 25, 30, 35, 40 and 45°C, respectively for 9 days is presented in the following Figures (3). Antimicrobial activity of metabolites by *B. megaterium* and *B. licheniformis*, maximum activity was observed at 30°C whilst the lowest

activity was recorded at 45°C. With *B. subtilis* was highest at 25°C whilst the lowest activity was observed at 45°C Figures (3). The higher hyphal growth inhibition of antimicrobial activity of metabolites by *B. megaterium* and *B. licheniformis* was at 30°C whilst *B. subtilis* was at 25 °C but the lowest inhibition was occurring at 45°C for the three strains.

Effect of initial pH on antibiotic production

To determine pH, the antimicrobial activity against *A. tumefaciens*, MRSA and *F. oxysporium* by *B. megaterium*, *B. licheniformis* and *B. subtilis* when the LB and PDB medium was initial pH values ranging from 4.0 to 9.0 was adjusted using different buffers and incubated at 30 °C for 9 days is presented in the following Figures (4). Maximum activity of antimicrobial metabolite for both isolates *B. megaterium* and *B. licheniformis* was observed at pH 7 with no activity at pH 4. Likewise there was no activity of the metabolites by *B. subtilis* at pH 4 but maximum activity was observed at pH 8.

Effect of carbon sources

The antibiotic activities of *B. megaterium*, *B. licheniformis* and *Bacillus subtilis*, against *A. tumefaciens*, MRSA and *F. oxysporium* as an indicator organism for bacteria plant pathogenic, human pathogenic and plant fungal pathogenic, respectively, when the fermentation medium was supplemented with various carbon sources glucose, galactose, xylose, sucrose, mannitol, lactose, starch, fructose, maltose and glycerol are presented in the following Figures (5, 6 and 7). For *B. megaterium*, the highest activity was observed when the medium was supplemented with starch (26, 31 mm and 15%) for *A. tumefaciens*, MRSA and *F. oxysporium*, respectively. For *B. licheniformis*, highest activity was observed with both starch and glycerol (Figures 5, 6 and 7). Whereas, other carbon sources were used in this study, they were associated with less antibiotic activity. For *Bacillus subtilis*, the highest activity was observed in the medium supplemented with glycerol (34 and 21 mm for *A. tumefaciens* and MRSA, respectively). No antibiotic activity against *A. tumefaciens* and MRSA was detected when the

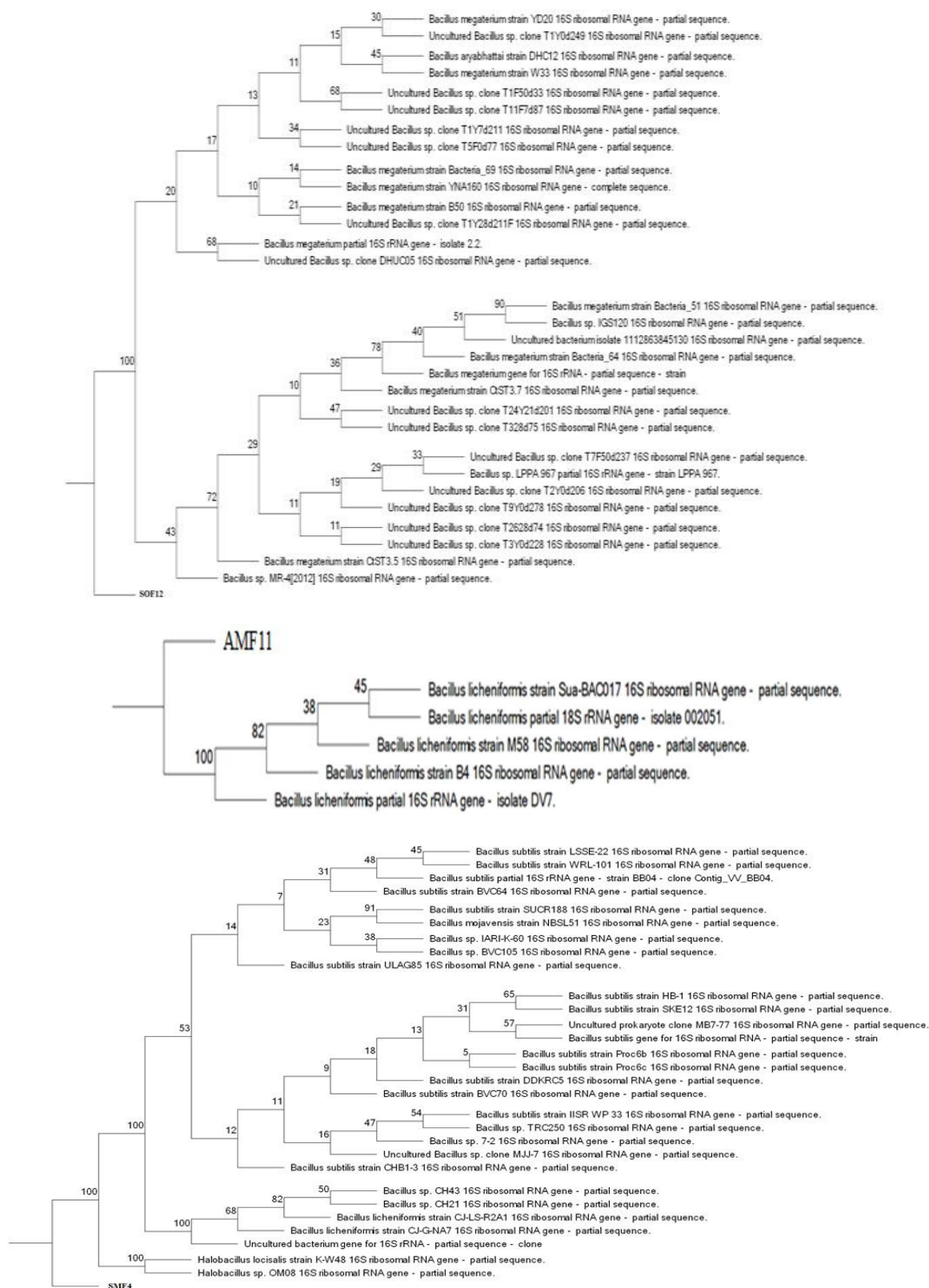


Figure 1: Phylogenetic tree of bacterial isolate SOF12, AMF11 and SMF4 relationship among the selected strains based on sequencing analysis and the most closely related bacterial species

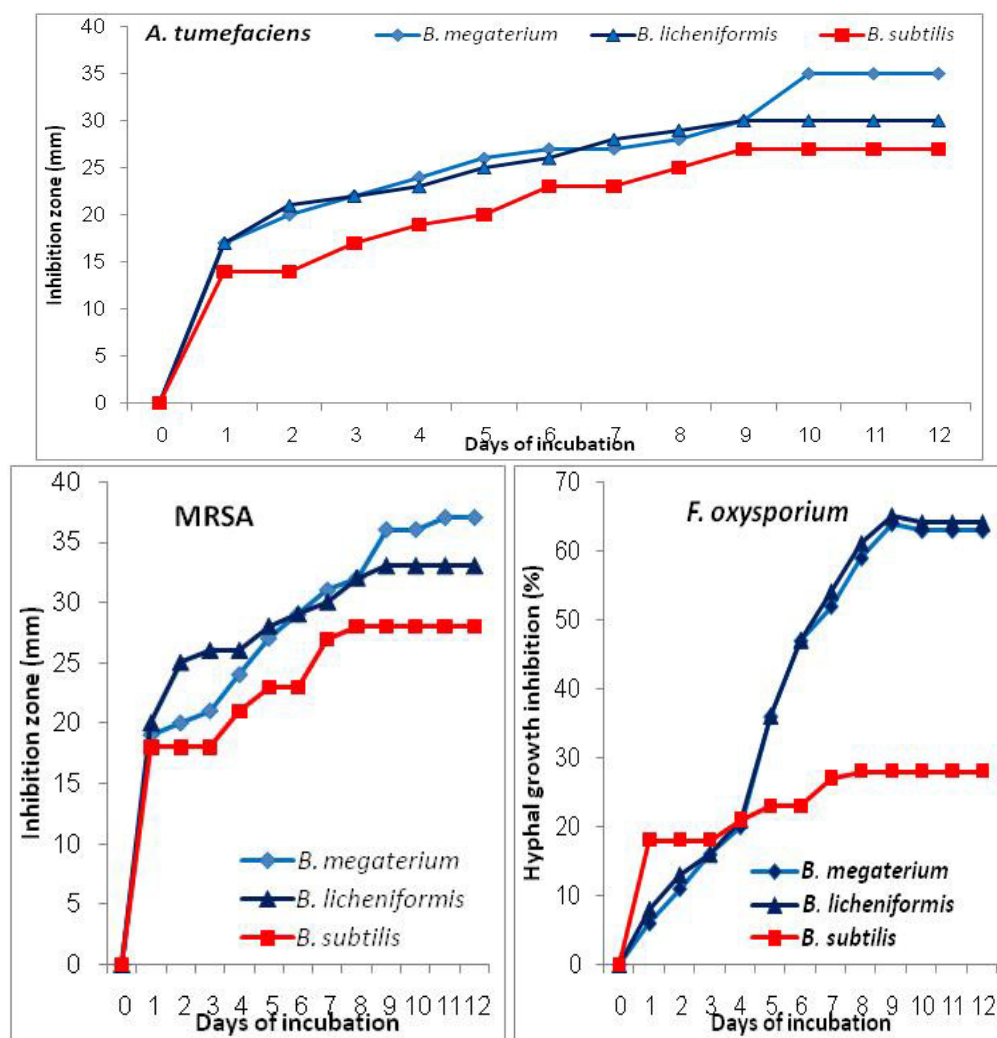


Figure 2: Mean of inhibition zone(mm) and percentage of hyphal growth inhibition of *B. megaterium*, *B. licheniformis* and *B. subtilis* against *A. tumefaciens*, MRSA and *F. oxysporium* under different incubation period

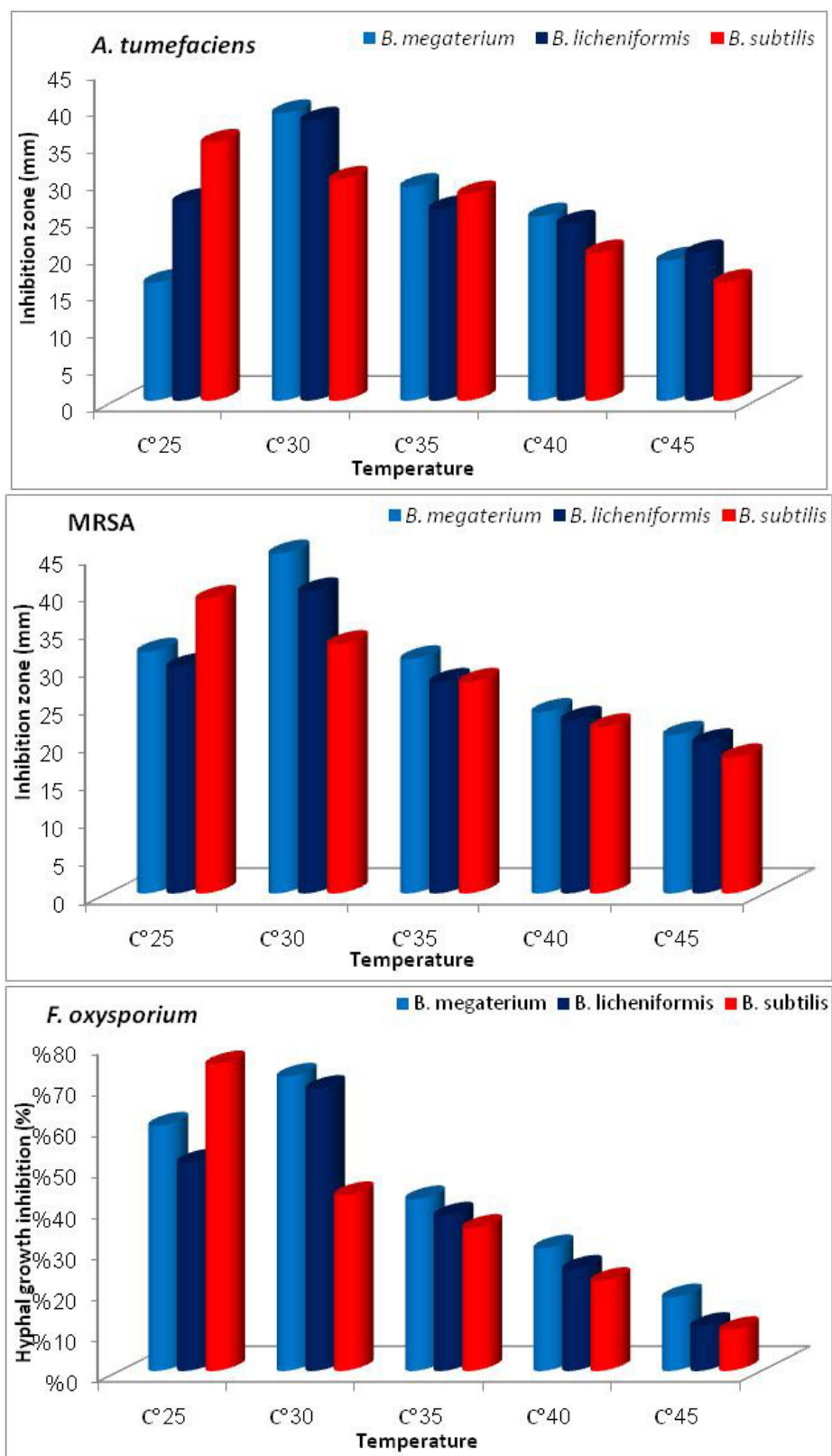


Figure 3: Mean of inhibition zone(mm) and percentage of hyphal growth inhibition *B. megaterium*, *B. licheniformis* and *Bacillus subtilis*, against *A. tumefaciens*, MRSA and *F. oxysporium* under different temperature degree

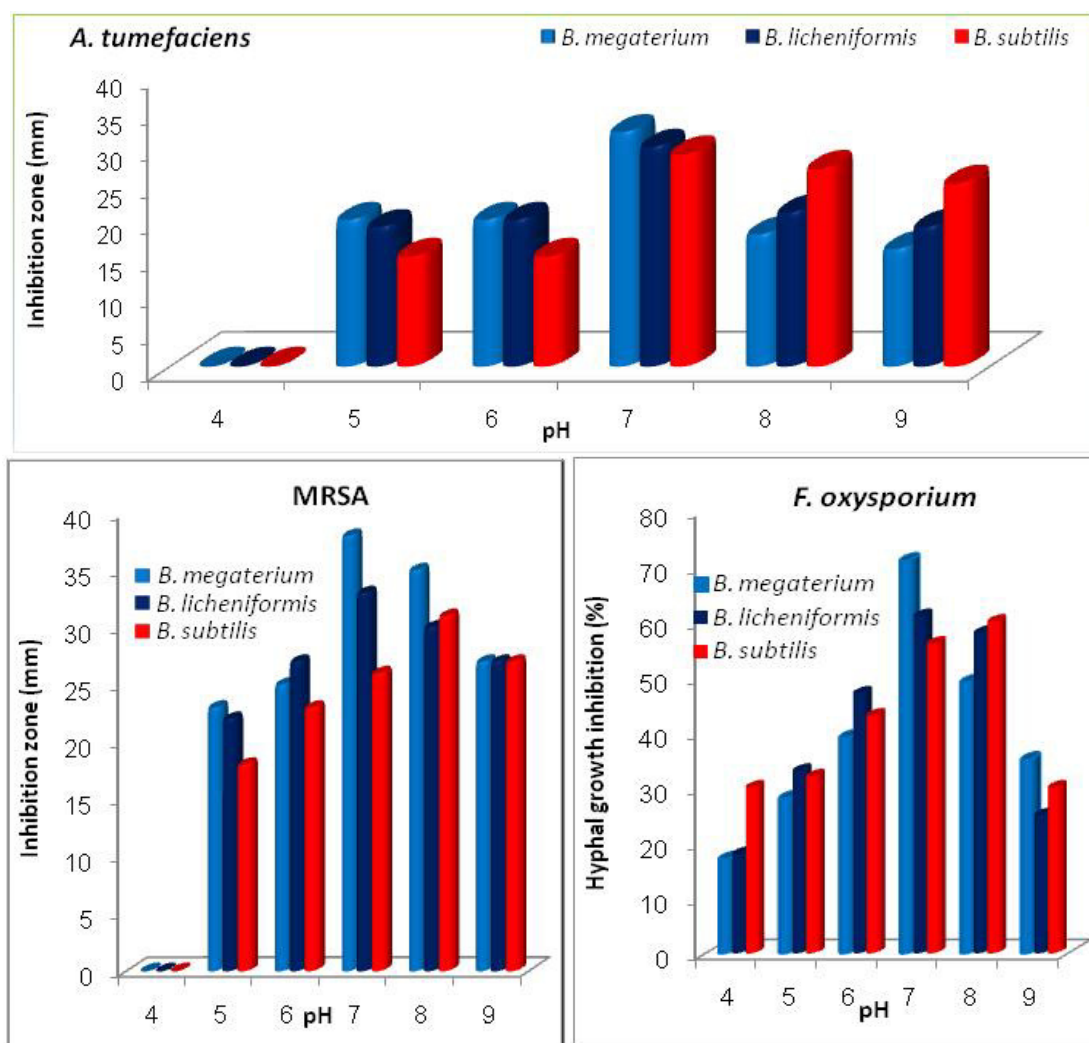


Figure 4: Mean of inhibition zone(mm) and percentage of hyphal growth inhibition *B. megaterium*, *B. licheniformis* and *Bacillus subtilis*, against *A. tumefaciens*, MRSA and *F. oxysporium*, respectively under different pH levels

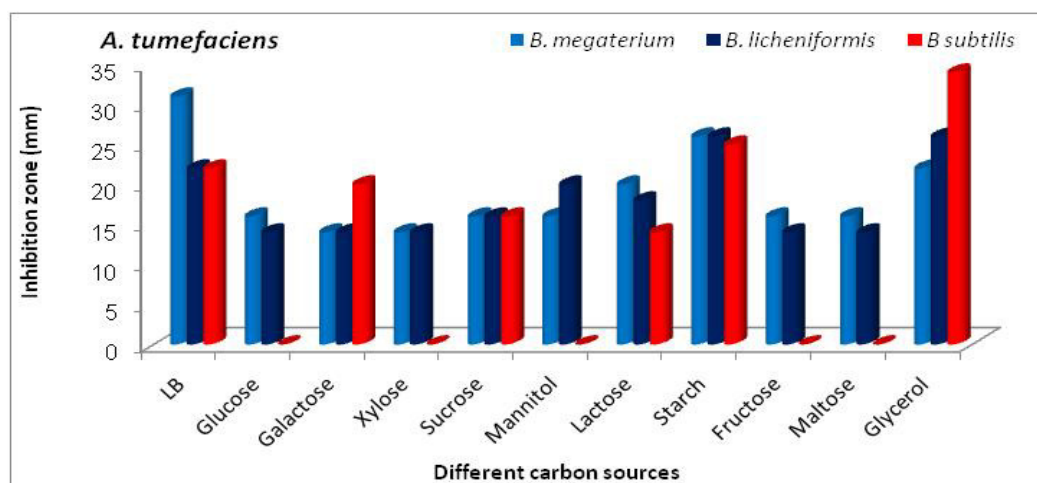


Figure 5: Mean of inhibition zone of *B. subtilis*, *B. licheniformis* and *B. megaterium* against *A. tumefaciens* under different carbon sources

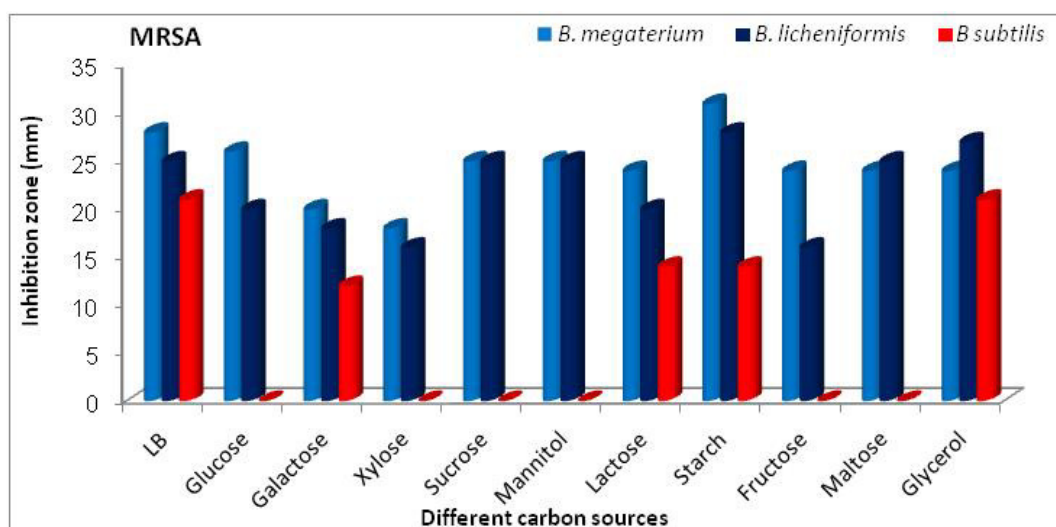


Figure 6: Mean of inhibition zone of *B. subtilis*, *B. licheniformis* and *B. megaterium* against MRSA under different carbon sources

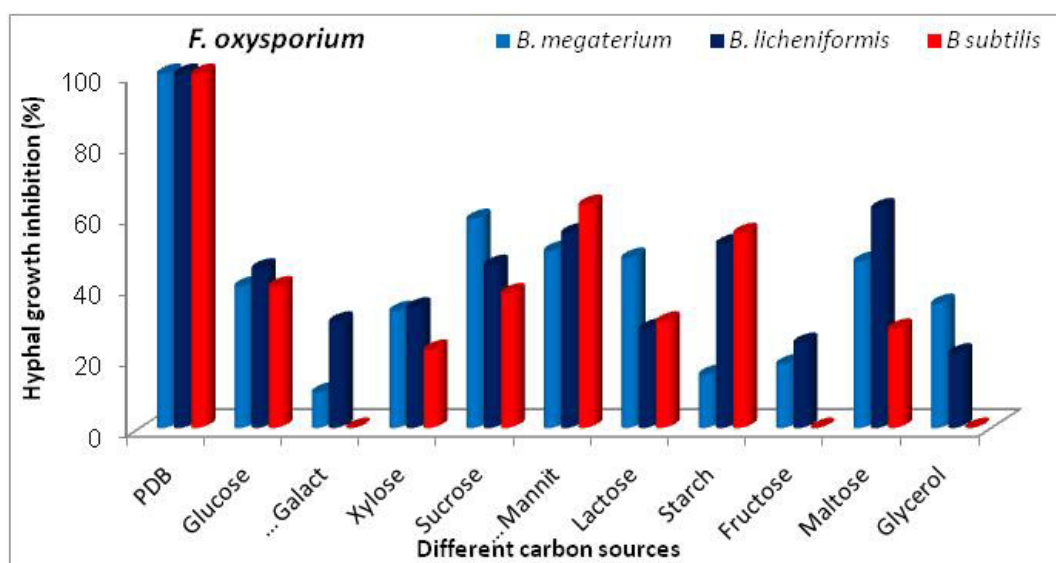


Figure 7: Percentage of Hyphal growth inhibition of *Bacillus subtilis* against *F. oxysporium* under different carbon sources

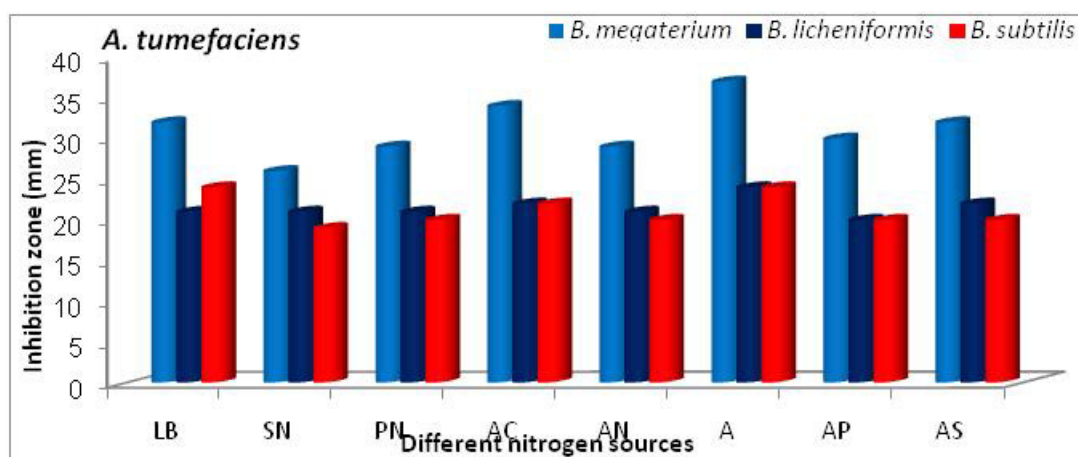


Figure 8: Mean of inhibition zone of *B. megaterium*, *B. licheniformis* and *B. subtilis* against *A. tumefaciens* under different nitrogen sources

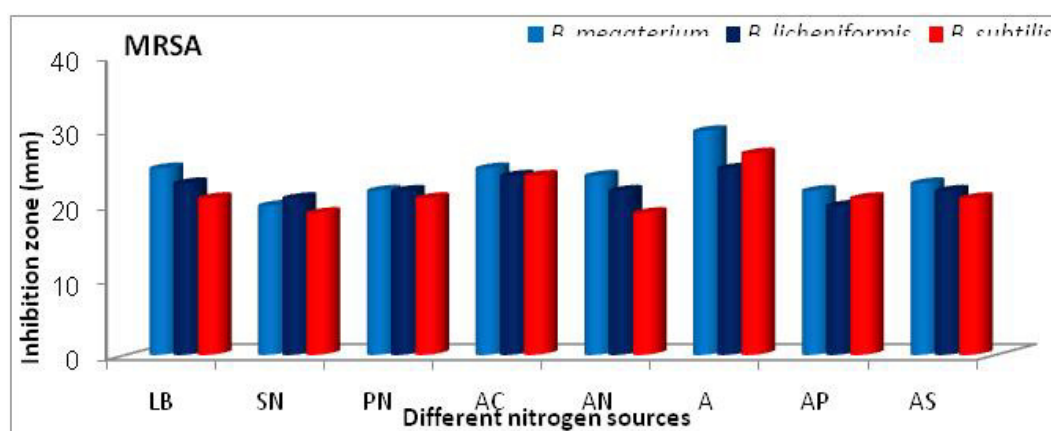


Figure 9: Mean of inhibition zone of *B. megaterium*, *B. licheniformis* and *B. subtilis* against MRSA under different nitrogen sources

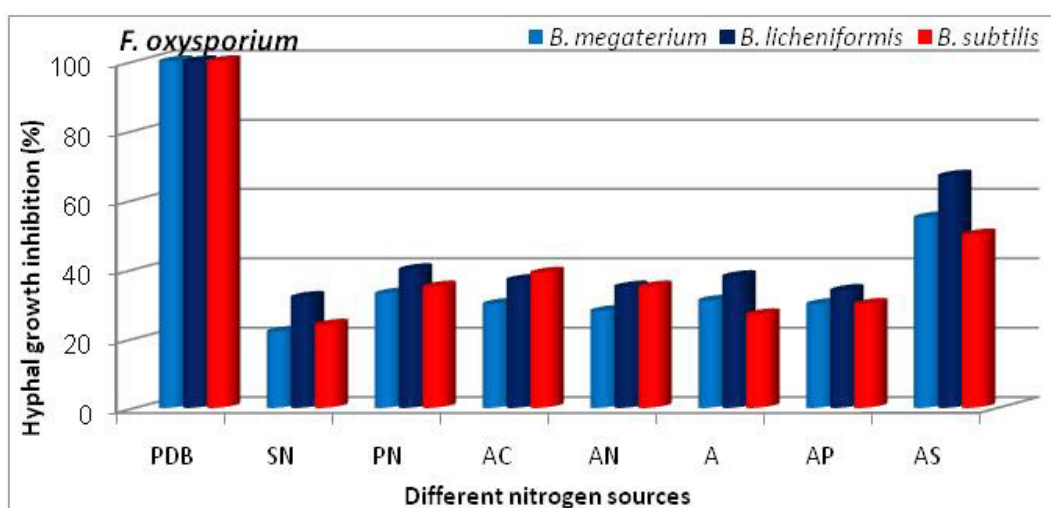


Figure 10: Percentage of hyphal growth inhibition of *B. megaterium*, *B. licheniformis* and *B. subtilis* against *F. oxysporium* under different nitrogen sources

medium was supplemented with glucose, xylose, sucrose, mannitol, fructose and maltose, neither was any activity observed against *F. oxysporum* when the PDA medium was supplemented with Galactose, Fructose and glycerol. Generally antibiotic activity against *A. tumefaciens* was greater than MRSA. In contrast to the activity of *B. subtilis*, the antimicrobial activity against MRSA and *A. tumefaciens* were greater than *F. oxysporum* for both *B. licheniformis* and *B. megaterium*.

Effect of Nitrogen Sources

The antimicrobial activity against *A. tumefaciens*, MRSA and *F. oxysporum* by *B. megaterium*, *B. licheniformis* and *B. subtilis* when the LB and PDB medium was supplemented with sodium nitrate (SN), potassium nitrate (PN), ammonium chloride (AM), ammonium nitrate (AN), asparagines (A), ammonium phosphate (AP) and ammonium sulphate (AS) is presented in the following Figures (8, 9 and 10). The highest antimicrobial activity by *B. megaterium*, *B. licheniformis* and *B. subtilis* occurred when the medium was supplemented with asparagine as nitrogen source (37, 24 and 24 mm, respectively). However, for *B. megaterium* and *B. subtilis* the least activity was produced by sodium nitrate (26 and 24 mm, respectively), whilst for *B. licheniformis*, the least activity was produced by ammonium phosphate (20 mm). For *B. licheniformis*, activity against MRSA was generally greater than against *A. tumefaciens* (Figures 8 and 9). For both *B. megaterium* and *B. licheniformis*, the activity against *A. tumefaciens* was much greater than MRSA whilst for *B. subtilis* the activity against MRSA was much greater than *A. tumefaciens* (Figures 8 and 9).

The highest hyphal growth inhibition of *F. oxysporum* occurred when PDA medium was supplemented with sodium nitrate as nitrogen source whilst the least activity was observed with the addition of ammonium sulphate (Figure 10). On the other hands, *B. megaterium* was highest hyphal growth inhibition of *F. oxysporum* than other *B. subtilis* or *B. licheniformis*.

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DISCUSSION

The result showed that, ethyl acetate crude antibiotic extract of three strains tested positive in bioassay against the all tested pathogenic bacteria while different between them. According to the ethyl acetate crude extract from

isolate SOF12 showed the highest active extracts followed by AMF11 and SMF4. These results indicated that the antagonistic activity was due to the production of an antimicrobial compound which can be extracted from the growth medium with organic solvents.

MIC of the crude extract ranged from 0.5µg/ml to 0.0625µg/ml. Isolate SOF12 highest activity against *S. aureus* and *Ps. Aeruginosa* with an MIC of 0.0625 µg/ml whereas the MIC for MRSA and *E. coli* was 0.125 µg/ml. This was further supported by the research conducted by (Kambezi and Afolayan2008) and (El-Mahmood2009) where hexane extracts are more active against gram-positive bacteria than gram-negative bacteria because gram-negative bacteria is more resistant to action of antibacterial agents. Therefore, some active biological substances did not dissolve easily in solvent. (Aouiche *et al.*, 2012) reported that isolate PAL111showed a strong activity against *C. albicans*, filamentous fungi, and Gram-positive and Gram-negative bacteria with MICs between 2 and 20 µg/ml for yeast, 10 and 50µg/ml for filamentous fungi, 2 and 10 µg/ml for Gram-positive bacteria and 20 and 75µg/ml for Gram-negative bacteria. (Valan *et al.*, 2008) reported that extract from actinomycetes isolate ERI-26 showed the MIC of 500 µg/ml against *C. albicans* while (Gandhimathi *et al.*, 2008) found that extract from *Streptomyces* sp. strain CPI 13 inhibited *C. tropicalis* with MIC and MFC values of 10 and 12.5 µg/ml, respectively.

Several factors influence production of secondary metabolites by microorganisms, the most important one being the composition of the production medium (Price-Whelan *et al.*, 2006). Knowledge of the effects of these factors helps to determine the optimum conditions for maximum metabolite production by the organisms. Variations in the fermentation media resulted in differences in the size of zones of inhibition produced which might be due to changes in pH during bacterial growth which in turn affected secondary metabolite production. (Roitman *et al.*, 1990) reported that by varying the conditions under which *Burkholderia cepacia* is grown, the yields and the composition of the antibiotic could be changed.

Based on the results obtained in this study, secondary metabolites production by *B. megaterium*, *B. licheniformis* and *Bacillus subtilis* is also strongly influenced by the medium composition and growth conditions. In the present study production of antibiotics was studied under different incubation periods (from zero to 12 days). It was found that antimicrobial activity increased generally from the first to the 9 day of incubation, after which the activity became stable. This study demonstrated that incubation time has a significant role in antibiotic production. Antibiotic concentration increased to a maximum at the mid-stationary growth phase and started declining at the end of this phase indicating it is synthesized as a secondary metabolite. It has been reported by (Haavik 1975) that bacitracin production by *Bacillus licheniformis* ATCC 14580

was observed only during the phase of rapid growth. The present study showed similar observation where maximum production was found during 48- 72 hour incubation, the phase of rapid growth for the *Bacillus* sp. whereas, according to (Egorov *et al.*, 1986) maximum efficiency of the bacitracin synthesis in case of *B. licheniformis* coincides with the end of the exponential growth phase and the onset of sporification. In submerged fermentation, 20 hours old vegetative inoculum gave the maximum yield of bacitracin by *B. licheniformis* (Yousaf, 1997). Production of secondary metabolites is generally associated with primary metabolite kinetics (De Vuyst *et al.*, 1996; Moretro *et al.*, 2000; Cladera-Olivera *et al.*, 2004) however, secondary metabolites production is recorded as a secondary metabolite in *Lactobacillus plantarum* LPCO10 (Jimenez-Diaz *et al.*, 1993), *B. licheniformis* 26L-10/3RA (Pattnaik *et al.*, 2001), *Lactococcus lactis* ssp. *lactis* (Cheigh *et al.*, 2002), *L. pentosus* B96 (Delgado *et al.*, 2005) and *Vibrio mediterranei* 1 (Carraturo *et al.*, 2008).

The biosynthesis of secondary metabolites by microorganisms is regulated by the growth temperature. Effect of different temperatures (25, 30, 35 and 45°C) on the activity of the metabolites was also studied. Each of the organisms had different temperatures at which the greatest antimicrobial activity was observed. Maximum antimicrobial activity was obtained at 30°C for *B. megaterium* and *B. licheniformis* and 25°C for *B. subtilis*. Temperature higher than the optimum resulted lowest activity was observed at 45°C. This clearly shows that organisms have specific temperatures or range of temperatures that enhance growth and secondary metabolite production. The effect of extent of incubation on the activity of the antibacterial compounds also showed that the activity of the antibacterial compound increase with increasing culture age up to a point (9 days in this study) where it becomes constant. Initial screening, in the present study, showed that maximum inhibition was observed by *B. megaterium* and *B. licheniformis* the strains at 30°C against all pathogenic microorganism used. Early studies by (Berdy 2005) have shown that maximum titers of secondary metabolites were obtained at 37°C after 3-5 days of incubation whereas; at 35°C maximum titer was usually attained after 120 hours of incubation. In agreement, Bizani and Brandelli (2002) and Sarika *et al.*, (2010) also reported that the amount of secondary metabolites production at 30°C was more significant than at higher temperatures. At higher temperatures and pH values, the production of secondary metabolites is lower because the energy used for maintenance purposes is much higher when the temperature or pH increases (Leroy and De Vuyst, 1999; Drosinos *et al.*, 2005).

Another important factor affecting secondary metabolites production is the initial pH of medium. The activity of several major enzymes that catalyze metabolic reactions of cell growth and antibiotic formation are affected by pH

(Guimaraes *et al.*, 2004). In general, the intracellular pH of most microorganisms is maintained near neutrality regardless of the pH in the outside medium (Garland, 1977). However as the proton gradient across the cytoplasmic membrane increases, the cells are forced to commit more of their resources towards maintaining the desired intracellular pH (Riebeling *et al.*, 1975), thus changes in external pH affect many cellular processes such as growth and the regulation of the biosynthesis of secondary metabolites (Chang *et al.*, 1991). Effect of pH was studied by adjusting the fermentation medium to different pH values. Antimicrobial activity of metabolites of SOF12, AMF11 and SMF4 which was identified as a *Bacillus* species was highest at pH 8. Similarly a study carried out by (Muaaz *et al.*, 2007) showed that *B. subtilis* produced more of an antimicrobial agent at a pH between 7 and 8. Another study carried out by (Awais *et al.*, 2008) reported that *B. pumilus* produced metabolites with maximum activity at pH 8 against *Micrococcus luteus*. Similar results have also been reported by (Moita *et al.*, 2005) in their study of inhibitory activity of *B. subtilis* 355.

Differences in pH requirements by organisms may be due to the fact that different strains of organisms are unique in their own way and therefore may require different conditions for production of antibiotics. All the organisms showed no growth when the pH of the medium was adjusted to pH 4, indicating their intolerance of acidic conditions. Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites (Chang *et al.*, 1991; Datta and Kothary, 1993; Solé *et al.*, 1994; Sole *et al.*, 1997). Effect of pH was studied by adjusting the initial pH (from 4.0 to 9.0) of the production medium. It is evident from the results that *B. subtilis* showed increased inhibition at pH 8, when tested against *A. tumefaciens* whereas, *B. megaterium* and *B. licheniformis* showed maximum zone of inhibition at pH 7. It has earlier been reported by Berdy (2005) that pH of 7.8-8 gave maximum production of bacitracin. Iglewski and Gerhardt (1978) have isolated a strain of *B. subtilis* with activity against *Proteus vulgaris* within the pH range of 5.7 to 6.8. It has been reported by Yousaf (1997) that optimum bacitracin yield from *B. licheniformis* was obtained with initial pH of 7.0.

Several studies have shown that the type and level of different carbon sources affect antibiotic production (Mataragas *et al.*, 2004; Drosinos *et al.*, 2005, 2006; Kanmani *et al.*, 2011). For *B. licheniformis*, highest activity was observed with both starch and glycerol. Whereas, other carbon sources were used in this study, they were associated with less antibiotic activity (Sole *et al.*, 1997) noted that glucose can be used as a source for bacterial growth while repressing the production of secondary metabolites. The organisms utilized glycerol and starch best for maximum production of the antimicrobial metabolites. Glycerol bioconversion in valuable products,

such as antimicrobial compounds, reduces production costs and environmental problems caused by accumulation of this waste (Da Silva *et al.*, 2009).

The nitrogen source also significantly affected antibiotic production (Mataragas *et al.*, 2004). Nitrogen is very vital in the synthesis of enzymes involved in primary and secondary metabolism (Merrick and Edwards, 1995). Therefore depending on the biosynthetic pathways involved, nitrogen sources may affect antibiotic formation. (Shapiro 1989) noted that the type of nitrogen source (organic or inorganic) plays a role in the synthesis of secondary metabolites. According to (Farid *et al.*, 2000) inorganic nitrogen sources such as nitrates, and several ammonium salts decrease the production of secondary metabolites by increasing cell biosynthesis. (Madhava and Gnanamani 2010) in their study on 'Condition stabilization for *Pseudomonas aeruginosa* MTCC 5210 to yield high titres of extra cellular antimicrobial secondary metabolite using "Response Surface Methodology" also reported that *Pseudomonas aeruginosa* MTCC 5210 utilized organic nitrogen source for better yield than the inorganic sources. These observations are consistent with the findings of this study as asparagine was better used for antibiotic production than the inorganic nitrogen sources used.

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