

Antimicrobial activity of *Bacillus circulans* isolated from rhizosphere of *Medicago sativa*Emad A Abada^{1,2*}, Hoda H El-Hendawy¹, Mohamed E Osman¹, Mohamed A Hafez³¹Botany and Microbiology Dep., Faculty of Science, Helwan university, Ain Helwan 11790, Cairo, Egypt.²Biology Dep., Faculty of Science, Jazan University, Jazan 114, KSA³Biology Dep., Faculty of Science, Al-Azahr University, Cairo, Egypt*Corresponding author: Tel.: +20225552468; E-mail: emadm_abada@yahoo.co.uk

Abstract: Microorganisms are good sources for the production of biologically active substances. The main purpose of this work is to study the antimicrobial and the chitinase activity of a bacterial isolate collected from *Medicago sativa* rhizosphere field of Helwan region, Cairo, Egypt. According to Bergy's manual of systematic bacteriology, the isolate was identified as *Bacillus circulans*. The antimicrobial activity of *Bacillus circulans* was studied against bacteria and fungi. Our results indicate the antimicrobial activity of *Bacillus circulans* against gram-positive, gram-negative bacteria and plant pathogenic fungi. The maximal antimicrobial activity was observed after 24 h at 30°C and pH 8. The best carbon and nitrogen sources was starch and DL-methionin respectively. The GC-mass analysis showed that the compound responsible for antimicrobial activity is 4-(Diphenylmethyl)-6 ethoxycarbonyl-1-phenyl-1H-pyranolo [4, 3-c] pyridine with molecular formula C₂₈ H₂₃ N₃ O₂. The Minimum Inhibitory Concentration (MIC) of C₂₈H₂₃N₃O₂ against Gram positive, Gram negative bacteria and unicellular fungi was in range of 0.5-2µg/ml. Interestingly, *Bacillus circulans* showed chitinase activity against different pathogenic plant fungi. Due to the antimicrobial and chitinase activity of *Bacillus circulans*, it could be used in industry for production of antibacterial compound and in biological control against different plant pathogen.

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Key words: *Bacillus circulans*, Antimicrobial activity, MIC, Chitinase.

1. Introduction

Microorganisms are good sources for the production of biologically active secondary metabolites substances (Monaghan Tkack. 1990; Omura, 1986; Woodruff.1980). Bacteria belonging to the genus *Bacillus* are among the most widely distributed microorganisms have produced antibiotics in the soluble protein structure and that these antibiotics have been found to be cheaper and more effective, these microorganisms are preferable for commercial production (Priest, 1989 and Debavov, 1982). Also, Strains of *Bacillus* are known producers of bioactive cyclic lipopeptides (Konz *et al.*, 1999). *Bacillus* has been investigated for their ability to produce so called bacteriocin like inhibitory substance BLIS (Motta *et al.*, 2007). It has been reported that strains of *B. thuringiensis*, *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, *B. megaterium* and *B. cereus* produce BLIS (Stein, 2005; Gray *et al.*, 2006; He *et al.*, 2006; Lisboa *et al.*, 2006; Sharma *et al.*, 2006).

In addition, Fifty-one *Bacillus* isolates were characterized for their antagonistic activity against *Xanthomonas campestris* pv causal agent of black rot in cabbage (Wulff *et al.*, 2002). On the other hand, *Bacillus subtilis* showed a production of a large number of antibiotics, which are classified as non-ribosomal (Zuber *et al.*, 1993). The antagonism of eight *Bacillus* isolates were investigated against different

Xanthomonas campestris pv strains, antibiotics was positive for four *Bacillus* isolates against all against *X. campestris* pv strains (Leila Monteiro *et al.*, 2005).

In another study, wild type strains of genus *Bacillus* were screened for their antimicrobial activity. Two strains exhibited antimicrobial activity against *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Aspergillus niger* and were identified as *Bacillus polymyxa* and *Bacillus circulans* (Perez *et al.*, 1993).

An antibiotic compound called Permetin A was purified from the culture filtrate of *Bacillus circulans* AJ 3902. It showed *in vitro* activity against Gram-negative, Gram-positive and some anaerobic bacteria (Takahara *et al.*, 1979). Butirosin produced by *Bacillus circulans* is among the clinically important 2-deoxy streptomine containing amino glycoside antibiotics (Kudo *et al.*, 2005). Although, *Bacillus circulans* recorded its antimicrobial activity against Gram-positive, it exhibit poor antimicrobial activity Gram-negative bacteria (He *et al.*, 2001). Not only bacterial secondary metabolites have antimicrobial activity against the growth of microorganisms, but also some bacterial enzymes involved in such important phenomena eg. Chitinase. Bacteria produce chitinase to digest chitin as a carbon and energy source. The chitinases are classified as endochitinase, exochitinase (EC.3.2.1.14), β-N-acetylglucosaminidase, and

chitinase (EC.3.2.1.30), which degrade chitin and its derivatives. Endochitinase splits the chitin polymer internally, whereas exochitinase releases chitin from one end. β -N-acetylglucosaminidase releases N-acetylglucosamine (NAG) monomers from chitin, while chitinase hydrolyzes chitin to N-acetylglucosamine (Flach *et al.*, 1992; Kuddus *et al.*, 2013). It was reported that, a *Bacillus subtilis* isolated from the soil of corn crops was applied as a seed treatment in the field to decrease the Smut Incidence Percentage (SIP) while increasing maize productivity (Mercado *et al.*, 2014). In the present study, we isolated and identified a bacteria named as *Bacillus circulans* that show dual significant antibacterial and chitinase activity against bacteria and plant pathogenic fungi.

2. Materials and Methods

2.1. Bacterial strains and fungi

Escherichia coli, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia sp*, *Salmonella sp*, *Bacillus subtilis*, *Staphylococcus aureus* were kindly provided by Dr. Samer Eleboady, Botany and microbiology department, Faculty of science, AL-Azhar University. *Fusarium oxysporum* causal agent of *Phaseolus* wilt, *Fusarium sp* causal agent of Cumin wilt, *Verticillium* causal agent of Cumin wilt, *Rhizoctonia solani* causal agents of rot of *Potato* and *Alternaria solani* the causal agents of early blight of *Potato* were kindly provided by Dr. K. Abu-elyoser, Department of plant pathology, Faculty of agriculture, Asuit University.

2.2. Isolation and identification of bacteria

Bacterial samples were collected from soil rhizosphere of *Medicago sativa* grown in an open field at Farowk center, Helwan, Cairo, Egypt. Samples were obtained 5 to 7cm depth from the surface of the soil and kept in sterile plastic bags, then were transferred directly to the laboratory and air dried. 10 g of each soil sample was added to 40 ml sterilized distilled water in 250 ml conical flask, shaken well for 10 min and the soil suspension was allowed to stand for 1 min until suspension supernatant appears clear, then 10 ml of supernatant were added to 90 ml sterilized distilled water in 250 ml conical flask. Then 10 fold serial dilutions were prepared in sterilized distilled water up to 10^{-6} , and 0.1 ml from each dilution were spread on the surface of nutrient agar plate, plates were incubated at 30°C for 24 h. All the obtained colonies were isolated, purified and streaked on nutrient agar slopes, maintained at 4 °C subcultured monthly in nutrient agar (Mew *et al.*, 1976).

2.3. Determination of antimicrobial activity in culture supernatant

2.3.1. Agar diffusion Assay

In general, this method is based on the observation the inhibition of growth of microorganisms in plates.

Gram-positive, Gram-negative bacteria and unicellular fungi (yeast) were used in this test.

Briefly, the tested bacteria were cultivated in nutrient broth medium for 24 h and seeded in nutrient agar plates, while yeast was seeded on Dox agar medium.

After solidification of nutrient agar and Dox medium, holes were made in the center of media by cork-borer. 100 μ of the overnight bacterial isolate culture was loaded into the plates hole. The Petri dishes were kept in room temperature for 1 hr. The nutrient agar plates were incubated at 30 °C for 24 h, while the Dox agar plates were incubated at 28 °C for 48 h. The detection of a clear zone around the well is an indication of antimicrobial activities of the strains under study. To study the antifungal activity of the bacterial isolate against mycelial fungi, the plates are inoculated with a suspension of fungal spores (Beaur *et al.*, 1966; Klaenhammer 1988; Schillinger and Luke, 1989).

2.4. Optimization of the culture conditions for the production of the antimicrobial compound

In order to determine the optimal medium composition to increase the antimicrobial activity of our bacterial isolate various physical and chemical conditions were used including different incubation periods, temperature, pH, carbon and nitrogen sources. The antimicrobial activity of the supernatant was determined as mentioned previously.

2.5. Extraction and purification of antimicrobial compound

The antimicrobial compound was extracted by mixing equal volume of culture supernatant with different organic solvent including (n-butanol, petroleum ether, di ethyl ether, benzene, n-hexan, ethyl acetate, chloroform and methanol). The extracted solutions were separated by separating funnel after vigorously shaking for 30 min at room temperature. Then, the mixture was allowed to stand till complete separation to upper and lower phase was formed. All lower phases evaporated by rotary evaporator the viscous liquid after evaporation is used for assessing the antimicrobial activity by paper disk diffusion method. Finally, Purification of the antimicrobial agent was done by using Prokasko- England Column chromatography length 20 cm and diameter 2 cm. Methanol and chloroform with different concentrations are used as eluent mixtures by using Silica gel 60 GF 200 [MERK]. Each fraction are collected every 30 ml and then antimicrobial assay carried out for all fractions against *Staphylococcus aureus* (Hyun *et al.*, 1999).

2.6. Minimum inhibitory concentration MIC

The tested organisms suspensions were adjusted to the density of the 0.5 McFarland standard in glass tubes. Different weights of the purified antimicrobial substance range from 0.1 μ g up to 100 μ g

were applied. The tubes were incubated for 24 h at 37 °C (Yeo and Livermore, 1994).

2.6.1. Instrumental analysis

Ultraviolet spectrum was measured with a U-1600 spectrophotometer, measuring mode absorption, sample volume 0.5 ml and wave length range 190-1000 (shimadzu).

2.6.2. Infrared Nexus 670 FTIR/ spectrometer

It was measured on a potassium bromide pellet with range 4000- 500 cm^{-1}

2.6.3. Gas Chromatography Mass and Mass spectroscopy

GC/MS Finnigan mat SSQ 7000, Digital DEC 3000 with ionization mode E1 eV 70. Column length 30 cm filled with fused silica capillary column and carrier gas helium, flow rate 13 ps sample size 1 μ l.

2.6.4. Transmission electron microscope (TEM)

TEM studies of our isolate grown in NA medium for 24 h under static conditions were done to observe flagella. The bacteria was mounted on a copper grid coated with Formvar, Carbon, and were stained with 0.5% phosphotungestic acid, pH 4.0. They were then observed with transmission electron microscope (JEOL 1010) in the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University.

2.6.5. Chitinase activity of the isolate strain:

Bacterial isolate streaked on colloidal chitin agar medium LB-CA (Difco) this medium contained (g/l): Tryptone 10, Sodium chloride 5, Yeast extract 5, colloidal chitin 1, Agar 15 and then selected based on their ability to hydrolyze and grow on chitin plates due to secretion of chitinase. The plates were then kept for incubation at 37°C for 24 hrs. The zone of clearance was measured and the cultures giving significant results were selected for further analysis according to (Rebecca *et al.*, 2013).

3. Results and Discussion

3.1. Isolation and identification of the bacterial strain showing antimicrobial activity

11 bacterial strains isolated from the rhizosphere of *Medicago sativa* grown in open field at Helwan, Cairo, Egypt. One strain showed highest antimicrobial activity against various tested organisms (Fig 1, Table 1). The bacterial isolate showed antimicrobial activity against not only various Gram-positive, but also Gram-negative bacteria. Furthermore, a fungus such as *Candida albicans* was also inhibited. Thus, it showed a very broad spectrum of inhibition ranging from prokaryotes to some eukaryotes. The bacterial strain was selected and initially identified as gram positive, rod shaped bacilli. Transmission electron microscope study revealed its rod shaped morphology and revealed presence of pretrichously inserted flagella, thus it confirmed its motility (Fig. 2). In order to further investigate its morphology and biochemical properties, we observed that it is encapsulated motile that produce

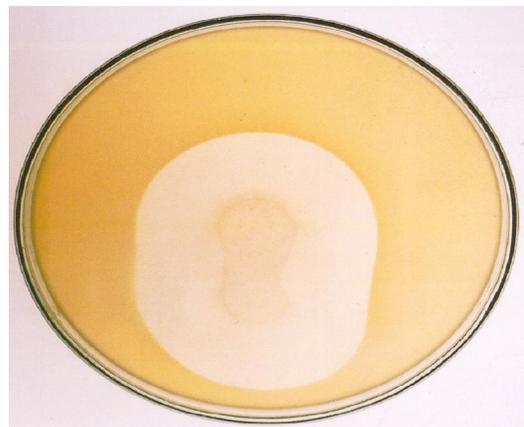


Figure 1. Antimicrobial activity of *Bacillus circulans* against Gram-positive and Gram-negative bacteria

Table 1. Antimicrobial assay of *Bacillus circulans* against human pathogenic test organisms

Test organisms	Inhibition Zone (mm)
A-Bacteria	
<i>Gram negative</i>	
<i>Escherichia coli</i>	13 mm
<i>Klebsiella pneumoniae</i>	12 mm
<i>Pseudomonas aeruginosa</i>	11 mm
<i>Salmonella typhi</i>	11 mm
<i>Serratia marcescens</i>	6 mm
Gram positive	
<i>Bacillus subtilis</i>	12 mm
<i>Staphylococcus aureus</i>	15 mm
B-Fungi	
<i>Candida albicans</i>	13 mm

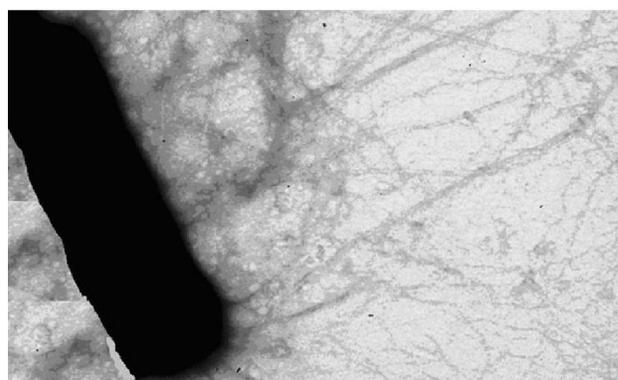


Figure 2. TEM scanning of *Bacillus circulans* showing pretrichously inserted flagella.

oval terminal spores. The vegetative forms occur singly and have rounded ends. On nutrient agar the organism gives a very mucoid adherent growth in 18 h at 37° C. In nutrient broth the growth is initiated much more slowly and a mucoid pellicle is formed. Acid without gas is produced from glucose, fructose, maltose, sucrose, galactose, raffinose and xylose. The Voges-

Proskauer test is negative. Gelatin and starch are hydrolyzed. Nitrites are produced from nitrates, as reported by (Smith *et al.*, 1946). The comparison of our bacterial isolate results with Bergey's manual of systematic Bacteriology results shown in (Tab 2).

Table 2. Identification of the strain *Bacillus circulans* N : Not found in Bergey's manual

Characteristics	Bergey,s manual	Bacterial isolate
Gram reaction.	+	+
Shape	Rod	Rod
Spore formation.	spore former	spore former
Catalase.	+	+
Nitrate reduction.	Partial	Partial
H ₂ S production.	+	+
Indole test.	—	—
Methyl red.	+	+
Voges proskauer.	—	—
Amylase	+	+
Lipase	+	+
Pectinase	+	+
Gelatinase	+	+
Raffinose.	+	+
Melibiose.	+	+
Starch.	+	+
L-rhamnose.	+	+
L-arabinose.	+	+
D-glucose.	+	+
D-xylose.	+	+
D-mannitol.	+	+
Maltose.	+	+
Sucrose.	+	+
Cellobiose.	+	+
L-arginine.	+	+
L-tyrosine.	—	—
L-alanine.	—	—
DL-tryptophan.	—	—
L-serine.	—	—
L-alanine.	—	—
DL-aspartic acid	—	—

N: not identified

According to Bergey's manual of systematic Bacteriology, The bacterial isolate was suggested to be *Bacillus circulans* (William and Wikins, 1986).

3.2. Optimization of the culture conditions for the production of the antimicrobial compound

3.2.1. Effect of Incubation periods on the antimicrobial activity

Incubation period is important parameter and also has economic value in production of antimicrobial compounds. Different incubations periods including (0, 6, 12, 18, 24, 36 and 48 h) were studied. The results showed that, the most effective incubation period is obtained after 24 h with inhibition zone of 13 mm with *Escherichia coli*, 17 mm with *Staphylococcus aureus* and 15 mm with *Candida albicans* (Fig. 3). It was suggested that incubation period at 24 h optimize the production of bacilysoicin produced by *Bacillus subtilis* (Tamehiro, *et al.*, 2002).

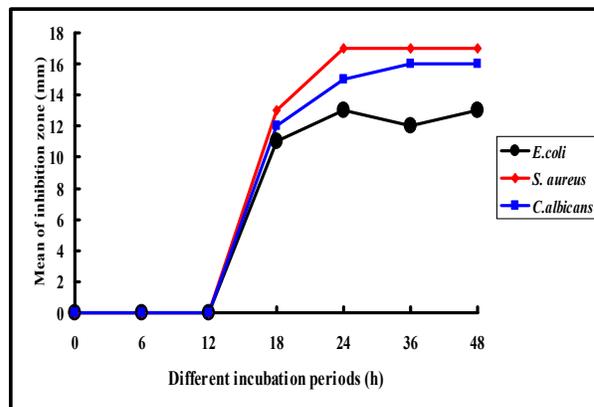


Figure 3. Effect of different incubation periods on the production of antimicrobial compound of *Bacillus circulans*.

3.2.2. Effect of temperature on the antimicrobial activity

Temperature is the very important physical factor which effects on the activity and behavior of bacterial cell. Effect of temperature on antimicrobial agents production showed that, the optimum temperature for production of antimicrobial agent of *Bacillus circulans* is 30 °C and it consider as mesophilic organisms because optimum temperature is ranged in temperature of mesophilic microorganisms 20 °C to 45 °C. The minimum temperature that *Bacillus circulans* can appear biological activity is 20 °C and the maximum temperature is 45 °C after or before these temperatures *Bacillus circulans* does not appear any biological activity (Fig. 4).

3.2.3. Effect of pH on the antimicrobial activity

The optimum pH value for the production of high yield of antimicrobial substance is important. Different pH values (2, 4, 6, 7, 8, 9 and 10) were studied. The most effective pH value for production of antimicrobial substance of *Bacillus circulans* is pH 8 which showed inhibition zone of 17 mm with *Escherichia coli* 21 mm with *Staphylococcus aureus* and 21mm with *Candida albicans* (Fig. 5). The specific pH range of antimicrobial activity of *Bacillus circulans* is ranged between pH values 6 to 9. It was reported that the pH 8 is optimal for kanamycin

synthesis (Ketaki and Majumdar, 1973). On addition, the antibiotic production of Rifamycin B was maximum at pH 8.0 and at 28° C (Mahalaxmi *et al.*, 2008).

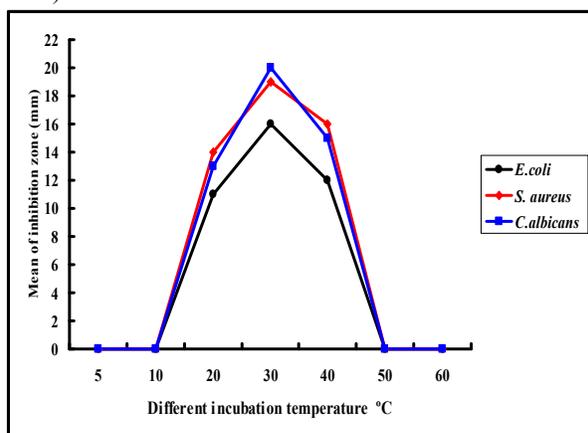


Figure 4. Effect of different temperature on the production of antimicrobial compound of *Bacillus circulans*.

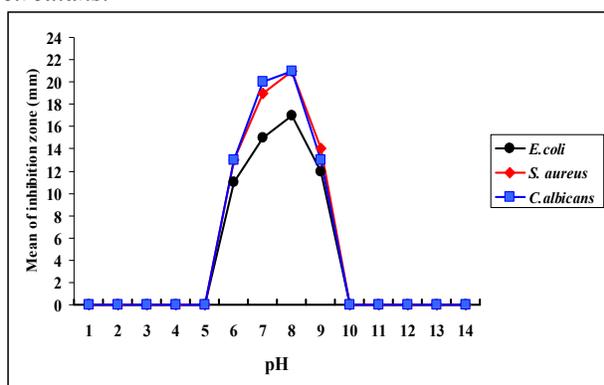


Figure 5. Effect of different pH on the production of antimicrobial compound of *Bacillus circulans*.

3.2.4. Effect of carbon source on antimicrobial activity

Different carbon sources such as monosaccharide D-Glucose, L-arabinose, D-fructose, D-raffionse, disaccharide maltose and sucrose, polysaccharide starch and finally inorganic carbon source sodium citrate were used. The best carbon source for the production of antimicrobial substance of *Bacillus circulans* was starch with inhibition zone of 15 mm with *Escherichia coli*, 18 mm with *Staphylococcus aureus* and 16 mm with *Candida albicans*. D-raffionse enhanced the production of antimicrobial substance but with less inhibition zone (Fig. 6). Studies on penicillin and actinomyces biosynthesis showed that a slowly utilizable carbohydrate gave a higher yield of the antibiotic because of its availability in adequate amount during both the phases of cellular growth and antibiotic production (Katz and Sivak, 1958; Koffler *et al.*, 1945). It was observed that Starch is the best carbon source on

the antimicrobial substance production by *Corynebacterium xerosis* NB-2(El-Banna, 2007).

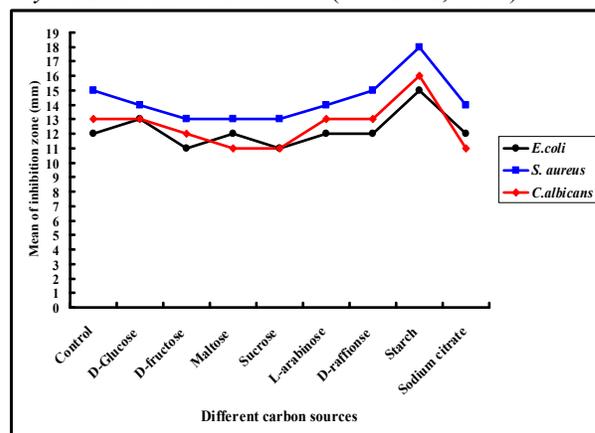


Figure 6. Effect of different carbon sources on the production of antimicrobial compound of *Bacillus circulans*.

3.2.5. Effect of Nitrogen source on antimicrobial activity

Nitrogen source is a very important factor in increasing bioactivity of activity of antimicrobial agent of *Bacillus circulans*. So, different nitrogen sources are introduced to *Bacillus circulans* L-valine, L-phenylalanine, DL-methionine, L-arginine, L-asparagine, L-tyrosine, L-alanine, urea, ammonium nitrate and DL-tryptophan to determine the best nitrogen sources. DL-methionine is the most effective nitrogen source for antimicrobial agent of *Bacillus circulans* (Fig. 7). The effect of DL-methionine increases the biosynthesis of cephalosporin-C and also, it is observed that the production of cephalosporin-C increases with increase in the methionine concentration up to 4.0 g/L and thereafter started decreasing. It has been reported that methionine at certain level optimum concentration stimulates the differentiation of mold and increases the yield of cephalosporin-C (Nigam, *et al.*, 2007).

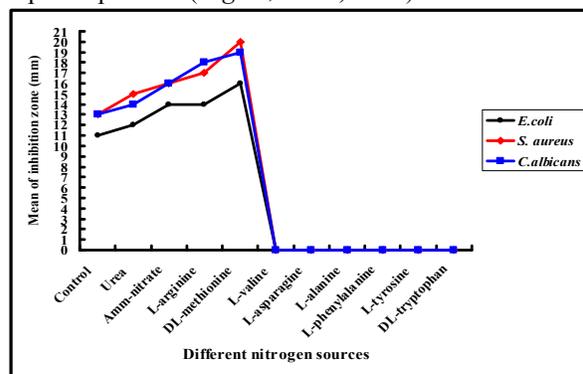


Figure 7. Effect of different nitrogen sources on the production of antimicrobial compound of *Bacillus circulans*.

3.2.6. Extraction and purification of antimicrobial compound

The use of different organic solvents is very important tools to extract the antimicrobial compound from bacterial media. Different organic solvent are used such as (n-butanol, petherium ether, di ethyl ether, benzene, n-hexan, ethyl acitate, chloroform and methanol). The most effective solvent which gave the higher inhibition zone from eight organic was n-hexane. Extraction of antimicrobial agent by n-Hexane was reported by (Nelis *et al.*, 1991). Purification of antimicrobial agent is carried out by mixture of methanol and chloroform as do by (Hyun *et al.*, 1999).

3.3. Minimum Inhibitory Concentration

The MIC of the compound was 1µg/ml against *Bacillus subtilis*, 2 µg/ml for *E.coli*, 0.5µg/ml for *Candida albicans* and was 1µg/ml for *Staphylococcus aureus* (Table.3).

Table 3: The MIC test of antimicrobial compound of *Bacillus circulans*

Test organisms	MIC (µgml ⁻¹)
<i>Escherichia coli</i>	2
<i>Bacillus subtilis</i>	1
<i>Staphylococcus aureus</i>	1
<i>Candida albicans</i>	0.5

It is evident that the active metabolite 4-(Diphenylmethyl)-6 ethoxycarbonyl-1-phenyl-1H-pyranolo [4, 3-c] pyridine showed significant antibacterial activity. The results of this assay strongly support that the isolated metabolite may be used in the management of microbial infection. In recent years the pathogenic organisms are gaining resistance to existing antimicrobial agents hence the search for new, safe and more effective antimicrobial agents is a pressing need. Thus the findings of this investigation would give valuable support to make clinical trial as well as toxicity studies of the isolated antimicrobial metabolite to get a more potent antimicrobial agent.

3.4. Spectroscopic characterization of the antimicrobial compound

The ultraviolet spectral analysis of the antimicrobial agent exhibit a maximum absorption peak wave length 200 nm (data not shown). The infra-red spectrum showed characteristic peaks at 3390 and 3072 mixed of stretching and interchain bonds of NH group with H- pond. The peaks at 1655, 1623 and 1323 showed C=O of peptide, deformation of NH group and amid III bond. Finally, 1177, 1107, 1074, 885, 852, 673, 653 and 617 showed skeletal vibrations of CH. Moreover, The Gas Chromatography Mass and Mass spectroscopy analysis appears that, the name of antimicrobial agent is 4-(Diphenylmethyl)-6 ethoxycarbonyl-1-phenyl-1H-pyranolo [4, 3-c] pyridine with a molecular formula C₂₈ H₂₃ N₃ O₂ and with Probability 85.65 (Fig. 8).

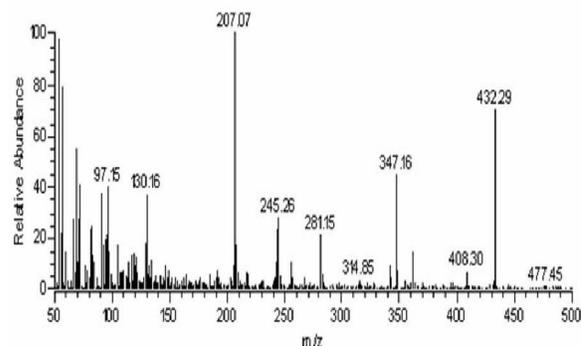


Figure 8. Gas Chromatography Mass and Mass spectroscopy analysis of antimicrobial compound of *Bacillus circulans*

3.5. Antifungal and Chitinase activity of *Bacillus circulans* against plant pathogenic fungi

Bacillus circulans showed positive chitinase activity (data not shown). Also, the antagonistic activity of *Bacillus circulans* were tested against some plant pathogenic fungi. Our data showed that there are an inhibition of growth for *Fusarium oxysporum* the causal agents of phasolus wilt by 33 %, *Fusarium sp* the causal agents of cumin wilt by 22 %, *Verticillium sp.* the causal agents of cumin wilt by 34 %, *Rhizoctonia solani* the causal agents of rot of potato by 55 % and *Alternaria solani* the causal agents of causal agents of early blight of potato by 45 %, respectively (Fig. 9).

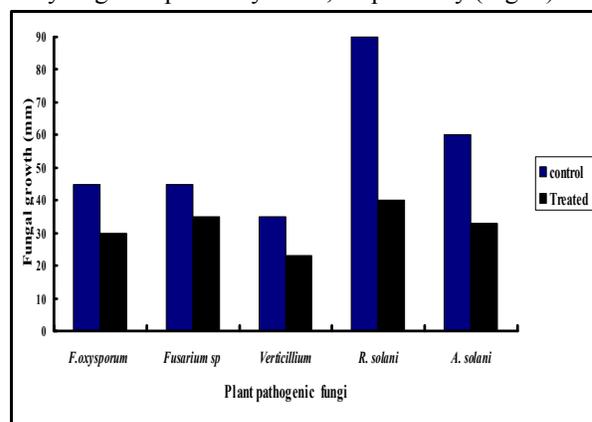


Figure 9. Antagonistic and Chitinase activity of *Bacillus circulans* against plant pathogenic fungi

The effect of Chitinase and antagonistic activity of *Bacillus circulans* against *Fusarium sp* (Fig 10), the causal agents of cumin wilt is studied under Transmission Electron Microscope. Observation with the TEM showed that the cell wall and septa of *Fusarium sp* (the control) were uniform. The hyphal cell was enclosed by a distinct electron-opaque cell wall, septum and normal nucleus shape (Fig. 11). The fungal cell exposed to *Bacillus circulans* growth become plasmolysed, the cell wall shrink, the fungal

nucleus was abnormal in shape and the septum became disordered (Fig. 12). Many *Bacillus sp* are known to suppress several fungal pathogens growth such as *Rhizoctonia*, *Sclerotinia*, *Fusarium*, *Gaeummanomyces*, *Nectria*, *Pythium* and *Phytophthora* (Fiddman *et al.*, 1994). Moreover, another *Bacillus sp.* produced a range

of metabolites including chitinases and other cell wall-degrading enzymes (Sadfi *et al.*, 2001). Several other workers have also found the biocontrol activities of *Bacillus* against many common phytopathogens (Chung *et al.*, 2008; Gajbhiye *et al.*, 2010).

However, it is likely that the most effective biological control strains act via multiple mechanisms.

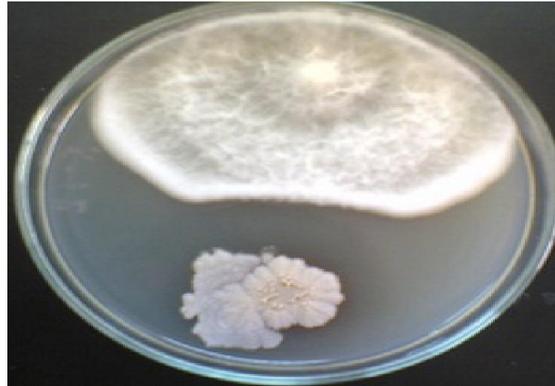


Figure 10. Chitinase and antagonistic activity of *Bacillus circulans* against *Fusarium sp* the causal agents of cumin wilt

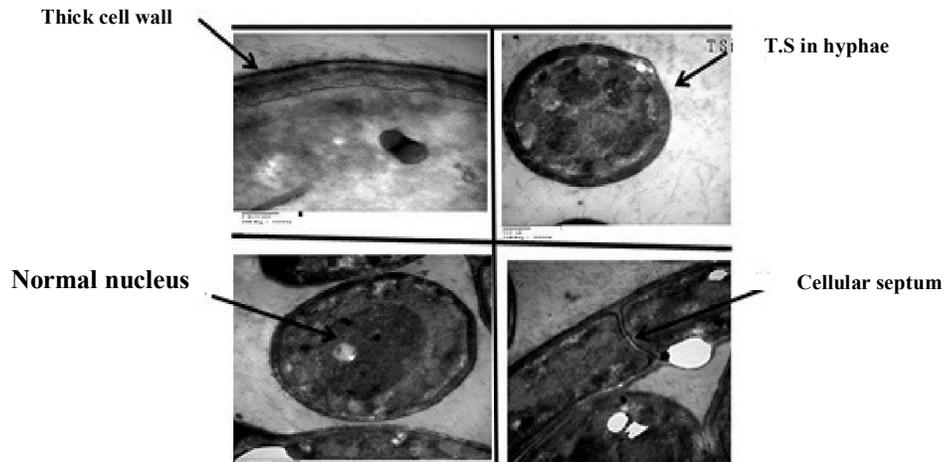


Figure 11. TEM study of the normal cell of *Fusarium sp* before exposure to *Bacillus criculans*

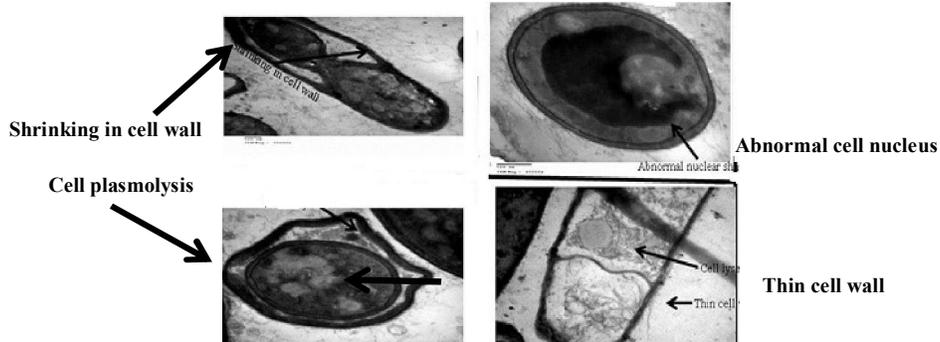


Fig. 12. ETM study of *Fusarium sp* exposed to *Bacillus circulans*.

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