The antimicrobial effect of *Bacillus spp* filtrates and extracted compound in some pathogenic agent

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Abstract

The study includes isolation and identification of .*Bacillus spp* samples using different cultural characteristics, physiological and biochemical tests .and VITEK 2 bacterial identification system to confirmed the identification . The results obtained that only 16 isolates of *Bacillus spp.* were isolated from 50 soil samples which represent 32% in percentage, those isolates were *Bacillus subtilis* 9 isolates 56.25%, *Bacillus firmus* 3 isolates 18.75% and *Bacillus atrophaeus* 4 isolates 25% . examined the antimicrobial activity by using filtrates of 16 *Bacillus spp.* isolate against tested bacteria included *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Vibrio cholerae, Klebsiella pneumoniae* and pathogenic fungal *Candida albicans, Candida tropicalis, Aspergillus fumigatus, Cryptococcus neoformance* isolates .The results showed that three isolates of *Bacillus subtilis* B1, B2, B5 showed antimicrobial activity against tested pathogenic bacteria and fungi compare to other *Bacillus* isolates . The isolate B5 showed higher activity among all the isolates, the higher activity was determined against *Staphylococcus aureus* and the lowest activity against *Salmonella typhi* . This isolate B5 had the same activity with fungi when recorded higher inhibition zone diameter against *Candida albicans* , The effect of the extracted crude *Bacillus subtilis* isolates B1, B2, B5 by using ethyle acetate showed higher activity than the filtrates . B5 isolate showed highest effects 24mm against G+ve *Staphylococcus aureus* and 19mm against G-ve *E. coli*, While the lowest effects was 16mm by isolate B1 on Salmonella typhi, and in fungi B5 isolate had higher activity on *Candida albicans* 27mm and lowest inhibition zone recorded by B2 isolate was 20mm on *Cryptococcus neoformans* .

Introduction

Bacillus is one of the well-studied and characterized genus of Gram-positive bacteria. The interest in this genus is due to its ability to form endospores, and to produce metabolites of interest in the agronomic, pharmaceutical and industrial fields. Bacillus is a complex genus at the genotypic, phenotypic, metabolic, taxonomic, and ecologic level, allowing them to be very versatile in different environments, especially in soil (1) .

Treatment of infectious disease with multidrug resistant strain of bacteria and fungi are becoming a major problematic in the whole world. Screening for new antibiotic from natural sources is becoming progressively important for the pharmaceutical industry as pathogenic bacteria and fungi are suggestively becoming resistant to generally used therapeutic agent. Antibiotic production is a feature of several kinds of soil bacteria .Of the several hundred naturally produced antibiotics that have been purified, only a few have been appropriately non-toxic to be of use in medication. Those that are currently of extreme use have been divided from a comparatively small group of microorganisms belonging to the genera Streptomyces, Penicillium, Bacillus, Micromonospora and Cephalosporium (2) .In recent years, many investigation have been carried out to isolate different strains of terrestrial Bacillus and identify their inhibitory compounds (3) .The aim of the present study was Studding the inhibitory effects of filtrate and extracted compound of Bacillus spp. on some human pathogenic bacteria and fungi .

Materials and Methods

Isolation and Identification of Bacillus Isolates :

After the incubation period of soil sample , bacillus isolates were characterized using different cultural characteristics, physiological and biochemical tests prescribed in Bergey□s Manual of Systematic Bacteriology (4,5) .and confirmed the identification by VITEK 2 bacterial identification system .

Preparation the filtrates of Bacillus isolates :

The primarily screened for Bacillus species isolates which have antimicrobial activity were done by inoculating in 100 ml nutrient broth and incubating at 30 ˚C for 72 hours in an orbital shaker at 150 rpm . After incubation , the culture was centrifuged at 6000 rpm for 15 min. to remove cell debris, then filtered through Millipore filter 0.22 µm unit .

Agar well diffution method was used in this test . About 100 µl of filtrate were loaded in to the wells of agar plates inoculated with the test microorganisms and blank well (fill with distilled water only) were used as a negative control . The plates were kept in room temperature for 1 hours and incubated at 37 ˚C for 24 hours (test bacteria) and for 5 days (test fungi) . After incubation period the inhibitory zones (mm) were measured .(6)

Extraction of active compound from Bacillus subtilis isolates :

Active *B. subtilis* isolates were grown in N. broth in 24 h. /37˚C. Then filtrates of 3 isolates were prepared as described above. The filtrates of 3 *Bacillus subtilis* isolates (B1 , B2 , B5) were collected and mixed with equal volume of ethyle acetate solvent in separation funnel and then shake gently . The organic phase were collected and re extracted with ethyl acetate until obtained extract purity with highly concentration. Then dried at room temperature . The yield from extract was dissolved in to ethanol for determination of antimicrobial activity (7) . Agar diffusion assay was used for this test . 100 µl of the obtained extracts were loaded in to the wells of agar plates inoculated with the test microorganisms and blank well (fill with solvent only) were used as a negative control . The plates kept in room temperature for 1 hour and incubated at optimum cultural conditions . the inhibitory zones (in mm) were measured .(8)

Result and discussion

This study included isolation and identification of Bacillus spp. from 50 soil samples collected preliminary cultured on nutrient agar media after making serial dilution in order to obtained primary Bacillus isolates, colony characterization, Physiological and Biochemical tests were performed to identify the bacteria as mentioned in 4,5. According to these tests only 16 isolates of *Bacillus spp* were isolated which represent 32 % in percentage, those isolates were:*Bacillus subtilis* 9 isolates 56.25 %, *Bacillus firmus* 3 isolates 18.75 % and *Bacillus atrophaeus* 4 isolates 25 %. And confirmed by VITEC 2 bacterial identification system.

Determination of antimicrobial activity of *Bacillus spp*. filtrates :

The results showed that only three isolates of *Bacillus subtilis* B1, B2, B5 showed antimicrobial activity against tested pathogenic bacteria and fungi compare to other Bacillus isolates which wasn't appeared any inhibitory effect against bacteria and fungi Table 2, 3 .The isolate B5 showed higher activity among all the isolates, the higher activity was determined against *Staphylococcus aureus* the inhibition zone diameter was 11 mmand the lowest activity was 8 mm against *Salmonella typhi* .

This isolate B5 had the same activity with fungi when recorded higher inhibition zone diameter 13 mm against *Candida albicans*, while lowest activity was 8 mm against *Cryptococcus neoformans* by isolate B2 . Our results obtained was agreement with many researches, in a study by 9 , recorded that *B. subtilis* isolated from soil showed good activity against pathogenic bacteria and fungi .

The inhibitory effect of *B. subtilis* was mentioned against some genus of G +ve bacteria like *Staph. aureus*, *Strep. pyogenes* and yeasts like *C. albicans*, *Cryptococcus neoformans* 10 . In study done by Hei *et al*. (2006) showed the inhibitor effect of ultra – filtered concentration of *B. subtilis* isolates showed higher effectiveness against wide spectrum of 32 strains of bacteria examined in his research(11) . Kummer et al ., (2009) found that strain of *B. subtilis* MTCC-8114 isolated from salin soil had antimicrobial activity against fungi (Trycophyton and Microsporum) (6). Several studies reported that secondery metabolites produced from Bacillus spp. were more effective against G+ve bacteria, resistance of G-ve bacteria to this secondery metabolites, may be attributed to the low permeability of outer membrane and lipopolysaccharide barrier for these compounds (12 ,13) .
In this experiment we used ethyle acetate as solvent to extracted some active compound could be produced by *B. subtilis* isolates . 3 active isolates B1, B2, B5 extracted using ethyle acetate in separated funnel . The organic phase was dried by air and resuspended with methanol . The active of these crude compound was measured on the test bacteria and fungi, the result summarized in Table 4,5 .

The effect of the extracted crude of Bacillus subtilis isolates B1, B2,B5 showed higher activity than the filtrates B5 isolate showed highest effects 24 mm against G+ve *Staph. aureus* and 19 mm against G-ve *E. coli.*

While the lowest effects was 16 mm by isolate B1 on *Salm. typhi* and in fungi B5 had higher activity on *C. albicans* 27 mm and lowest inhibition zone recorded by B2 was 20 mm on *Cryptococcus neoformans*, Alshahrane *et al ., (* 2015) mentioned that the crude extracted of Bacillus spp. had good antimicrobial activity than the supernatant of Bacillus against some G+ve bacteria (*Staph. aureus*, *Micrococcus lates*)(14) Sirtori *et al* ., (2008) reported that the extracted of *B. subtilis* had good activity against *Listeria monocytogens* and *Enterococcus fecalis* (15). the effect of Bacillus to have inhibitory effect against the bacteria returned to ability of production different metabolites which have these activity (14) .

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Table (1) : Result of Morphological and Biochemical Characteristics of *Bacillus* isolates

Characteristics	*Bcillus atrophaeus*	*Bacillus subtilis*	*Bacillus firmus*
Shape	Rods	Rods	Oval
Gram staining	+	+	+
Motility	+	+	+
H2S production	-	-	-
Indole	-	-	-
Oxidase	-	-	+
Catalase	+	+	+
Urease	ND	-	-
Voges-proskauer	+	+	-
Hydrolysis gelatin	+	+	+
Hydrolysis ofstarch	+	+	+
Glucose	+	-	+
Lactose	-	+	-
Sucrose	+	+	+
Mannitol	ND	+	+

ND: not determined

Table (2) :Antimicrobial activity of *Bacillus spp* filtrates. against pathogenic bacteria

Bacillus isolate	Test bacteria [inhibition zones in mm]						
	S.aureus	*S.pyogens*	*E.coli*	*p.aeruginosa*	*S.typhi*	*V.cholerae*	*Klebsilla spp.*
BI	10	9	-	-	8	9	-
B2	10	9	9	9	-	-	-
B3	9	-	-	-	-	-	-
B4	-	8	-	-	-	-	-
B5	11	10	9	-	8	9	-
B6	-	-	8	-	-	-	-
B7	-	-	-	-	-	-	-
B8	-	8	-	-	-	-	-

B9	-	-	7	-	-	-	-
A1	-	-	-	-	-	-	-
A2	-	-	-	-	-	-	-
A3	-	-	-	-	-	-	-
A4	-	-	-	-	-	-	-
F1	-	-	-	-	-	-	-
F2	-	-	-	-	-	-	-
F3	-	-	-	-	-	-	-

Bacillus subtilis : (B1–B9), *Bacillus atrophaeus* : (A1 – A4), *Bacillus firmus* : (F1 – F3) .

Table (3) : Antimicrobial activity of *Bacillus spp.* filtrates against fungi

Bacillus isolates	Test fungi [inhibition zones in mm]			
	Candida albicans	*Candida tropicalis*	*Aspergillus fumigatus*	*Cryptococcus neoformans*
B1	11	9	10	-
B2	10	9	-	8
B3	-	8	-	-
B4	-	-	-	-
B5	13	10	11	10
B6	7	-	-	-
B7	-	7	-	-
B8	8	-	-	-
B9	-	-	-	-
A1	-	-	-	-
A2	-	-	-	-
A3	-	-	-	-
A4	-	-	-	-
F1	-	-	-	-
F2	-	-	-	-
F3	-	-	-	-

Table (4) : Effect of the extracted crude of *Bacillus subtilis* isolates (B1, B2, B5) against bacteria

Bacillus isolate	Test bacteria [inhibition zones in mm]						
	S.aureus	*S.pyogens*	*E.coli*	*p.aeruginosa*	*S.typhi*	*V.cholerae*	*Klebsilla spp.*
BI	21	19	-	-	16	17	-
B2	20	19	18	17	-	-	-
B5	24	21	19	-	18	18	-

Table (5) : Effect of the extracted crude of *Bacillus subtilis* isolates (B1, B2, B5) against test fungi

Bacillus isolates	Test fungi [inhibition zones in mm]			
	Candida albicans	*Candida tropicalis*	*Aspergillus fumigatus*	*Cryptococcus neoformans*
B1	24	21	22	-
B2	23	21	-	20
B5	27	22	24	23

الفعالية المضادة للاحياء المجهرية لرواشح ومستخلص بكتريا *Bacillus spp* في بعض المسببات المرضية

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الخلاصة

اجريت هذه الدراسة لعزل وتشخيص انواع*.Bacillus sp* من التربة والتحقق من فعاليتها المضادة للاحياء المجهرية ضد بعض المسببات المرضية البكترية والفطرية المعزولة من الانسان .

تضمنت هذه الدراسة جمع 50 عينة تربة لعزل *Bacillus* .شخصت عزلات *Bacillus* باستعمال مختلف الفحوصات المختبرية , الصفات الزرعية, الفحوصات الفسيولوجية والكيميائية , تم استعمال نظام Vitek 2 لتحديد البكتريا لتأكيد التشخيص .اظهرت النتائج ان 16 عزلة فقط من *Bacillus spp.* تم عزلها من 50 عينة تربة والتي بلغت نسبتها 32% متضمنة 9 عزلات *Bacillus subtilis* 56.25% , 3 عزلات *Bacillus firmus* 18.75% و 4 عزلات *Bacillus atrophaeus* 25% . اختبرت الفعالية المضادة للميكروبات باستخدام الرواشح من 16عزلة *Bacillus spp.* ضد البكتريا والفطريات المختبرة . كانت العزلات بكتيرية *Streptococcus pyogenes,Staphylococcus aureus* , *Vibrio cholerae* , *Salmonella typhi, Pseudomonas aeruginosa* , *Escherichia coli* , *Klebsiella pnumoniae* و4 عزلات فطرية *Candida albicans* , *Candida tropicalis* , *Cryptococcus neoformance* ,*Aspergillus fumigatus* . واظهرت النتائج ان ثلاث عزلات فقط *Bacillus subtilis* B1,B2,B5 اظهرت الفعالية المضادة للميكروبات ضد البكتريا والفطريات المرضية المختبرة مقارنة مع عزلات *Bacillus*الاخرى . اظهرت العزلة B5 فعالية عالية بين جميع العزلات . حددت اعلى فعالية ضد بكتريا *Staphylococcus aureus* كانت منطقة التثبيط 11mm وادنى فعالية كانت 8mm ضد *Salmonella typhi*. هذه العزلة B5 كانت لها نفس الفعالية مع الفطريات عندما سجلت اعلى قطر منطقة تثبيط 13mm ضد *Candida albicans* من حين اقل فعالية كانت 8mm ضد *Cryptococcus neoformans* بواسطة عزلة B2 .

اظهر تاثير الخام المستخلص من عزلات *Bacillus subtilis* B5,B2,B1 باستخدام خلات الأثيل فعالية اكثر من الرواشح . اظهرت العزلة B5 تاثيرات عالية 24mm ضد الموجبة لصبغة كرام *Staphylococcus aureus* و19mm ضد السالبة لصبغة كرام *E. coli* . بينما كان التاثير الاوطأ 16mm من خلال العزلة B1 على *Salmonella typhi* , و في الفطريات كانت العزلة B5 اعلى فعالية على *Candida albicans* 27mm وادنى منطقة تثبيط سجلتها العزلة B2 كانت 20mm على *Cryptococcus neoformans* .