Antifungal activity OF ethyl acetate extract of four species *Bacillus* isolated from soil

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ABSTRACT: During study isolated and identificated four species backed to genus of *Bacillus* from 30 soil samples,in Basrah province (Al-Hartha, Abu-Alkaseeb, Al-Jibasi and Safwan) by using dilution method. crude metabolites extract from bacterial isolates prepared by ethyle acetate extract for bacterial isolates. The crude metabolite extracted from *Bacillus stearothermophils* showed antifungal activity against both filamentous fungi and yeasts while extract from *B.licheniformis* Don't exhibited any antifungal activity against all tested.
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I. Introduction

Antibacterial substances are widely produced by a large number of microorganisms. Currently microorganisms of genera *Bacillus, Penicillium* and *Micromonospora* are known to produce more than 5000 different antibiotics[1]

Many species of *Bacillus* are capable of producing biologically active substances disintegrating fungal cell walls (*e.g.*, polymyxines, bacitracin, butirosin, thuricins and cereins). *Bacillus,* widespread naturally as non-pathogenic bacteria, can produce many different antimicrobial substances. The metabolites from *Bacillus* showed the activity against some kinds of fungi[2,3,4]

The object of this study was to characterize *Bacillus* strains GB-017 and GB-0356, which produce antifungal substances. We also investigated the culture conditions required for the production of antifungal and the properties of the antifungal substance produced by *Bacillus* spp. isolated from soil of Korea. The researcher were found to be bacillus-shaped, gram-positive and motile, and to inhibit *Botrytis cineria, Fusarium* sp., *Pythium* sp., and *Rhizoctonia solani*. Antagonistic activity was maintained up to pH 9.0, and the antifungal activity was stable to heat at 80oC for 1 h. [5]During this study evaluated the potential antimicrobial activity of a bioactive compound produced by a *Bacillus sp.* isolated from soil sample in Woods Hole Massachusetts and the result showed high antifungal against test fungi [6]

Antagonistic properties of the strain *Bacillus subtilis* IB-54 with respect to dermatophytes fungi *Trichophyton rubrum, T. mentagrophytes var. gypseum, and Microsporum canis* was studied. The studied strains of *Bacillus* sp. effectively inhibited growth and development of dermatophytes when were cultivated on the media containing different carbon sources. [7] (Lukmanova *et al.,* 2008).

Among different bacterial isolates, a potent *Bacillus subtilis* MTCC 8114 was isolated from garden soil samples which showed antifungal activity against test fungi i.e. *Microsporum flavum and Trichophyton sp.* [8]

Researcher to search for new and potent antimicrobial compound. Therefore the aim of our research is to the screening of potent antibiotic producing microorganism from the natural sources such as soil. The bacterial speciessuch as *Bacillu stearothermophilus, B.polymyxa, B.subtilis and B. licheniformis* well studied for the production of antibiotic.

II Materials and methods

Sample collection

30 Soil samples from different places of the Basrah Basrah province (Al-Hartha, Abu-Alkaseeb,Al-Jibasi and Safwan) were collected in the sterile small reagent bottles from the surface and labeled properly and stored at 4ºC until examination.

Isolation of pure colonies

Approximately 1 gram of soil sample was suspended in 9 ml sterile distilled water and vortexed vigorously to make uniform suspension. After that successive serial dilutions were made by transferring 1 ml of aliquots to 2nd test tube containing 9 ml of sterile water and in this way dilution up to 10^{-10} were made. An aliquot of 0.1 ml of each dilution was taken and spread evenly over surface of nutrient agar plates and incubated overnight at 37ºC.

After 24 hours plates were examined and few colonies nearly same in appearance showed [9]

Identification of *Bacillus* isolates

Isolate was characterized by morphological and biochemical analysis using the tests prescribed in [9].

1- Gram staining
2- Spore formatting
3- starch analysis(Amylase production)
4- Vogus proskauer test(VP)
5- Citrate
6- Growth at 55
7- Oxidase production
8- Catalase production
9- Motility

Extraction of crude antibiotics from four species of *Bacillus*

The primarily screened bioactive *Bacillus* isolates were inoculated into 100 ml glucose nutrient broth each only and incubated for 24 hours at 37ºC. After incubation, 10 ml of culture broth was transferred to another 100 ml of sterile broth in 250 ml conical flask and incubated at 37ºC for 3 days with shaking (120 rpm). Following 3 days incubation the broth culture was centrifuged at 10000 rpm at /5 mint. The culture supernatant was collected and mixed with equal volume of ethyl acetate solvent in a separating funnel and then shake gently for 2 hours. The organic solvent was collected and dried at room temperature. The solvent extraction was then assayed for antifungal activity by well agar diffusion method. The residue was termed as crude antimicrobial metabolite [1]

Test organisms

Eight fungal isolates were Isolated from clinical specimens in Al-Zubair hospital (Onychomycosis, Otomycosis, oral candidiasis) from periods 1-1-2013/1-4-2013 this fungal isolates identification depended on [10,11]

Antifungal assay

To test antifungal activities of the crude extract of isolates *Bacillus*, the well agar diffusion method[12]

Activated fungal isolates by re cultured on Sabourauds dextrose agar(SDA) (Himedia, India) incubated at 27 C for week to filamentous fungi and 3 days for yeasts. Spreading fungal isolates suspension at concentration $6*10^{8}$ by using MacFrland scale. The crude extract was dissolved in respective solvent to get a solution of known_concentration (100mg/ml). make (5 mm wells) were then Added 100 µl from the crude extract of four species to *Bacillus* were placed on SDA medium evenly seeded with the test fungi. and blank well(fill with solvent) were used as a negative control respectively. The plates were then kept in incubators at 27ºC for about 3 days for yeasts and 7 days for filamentous fungi.Finally the plates were incubated at this temperature to allow maximum growth of the fungi. The test materials having antifungal activity inhibited the growth of the fungi and a clear, distinct zone of inhibition was visualized surrounding the well on the medium. The antifungal activity of the test agent was determined by measuring the diameter of the zone of inhibition in terms of millimeter (mm) with a transparent scale and the experiment was carried out in triplicate.

II-Results and discussion

Screening for new antibiotics from natural sources is becoming increasingly important for the pharmaceutical industry as pathogenic fungi are increasingly becoming resistant to commonly used therapeutic agents[13]

Scientists are now working to explore alternative drugs from microbial sources to discover new drug as antifungal. Secondary metabolites from microorganisms having a diverse chemical structure and biological activities are produced only by some species of a genus *Bacillus* [14]

During this study The four species of Bacillus were identified on basis of morphological and biochemical characteristic (Table 1).

Also this study was performed with an aim of screening and isolation of antimicrobial substances producing bacteria from soil. Four *Bacillus* species were isolated from soil samples as they produced thin clear zone around the colony during well agar diffusion technique and purified by subculture. But one of them *Bacillus stearothermophilus* showed best activity against pathogenic fungi while *B. licheniformis* don't showed any activity against all tested fungi (Table 2, Plate (1,2)) 0. The results showed activity it as antifungal because these substances (crude metabolites) possess an active proteinaceous nature responsible for the activity[4]

The isolated species exhibited inhibitory effect on the growth of different test fungi, the crude secondary metabolites at a dose of 100 mg/ml of *Bacillus stearothermophilus* obtained from ethyl acetate extraction showed strong antifungal activity against Filmentouse fungi and yeast with the zone of inhibition range 6 to 27 mm (Table 2, Plate 1,2).

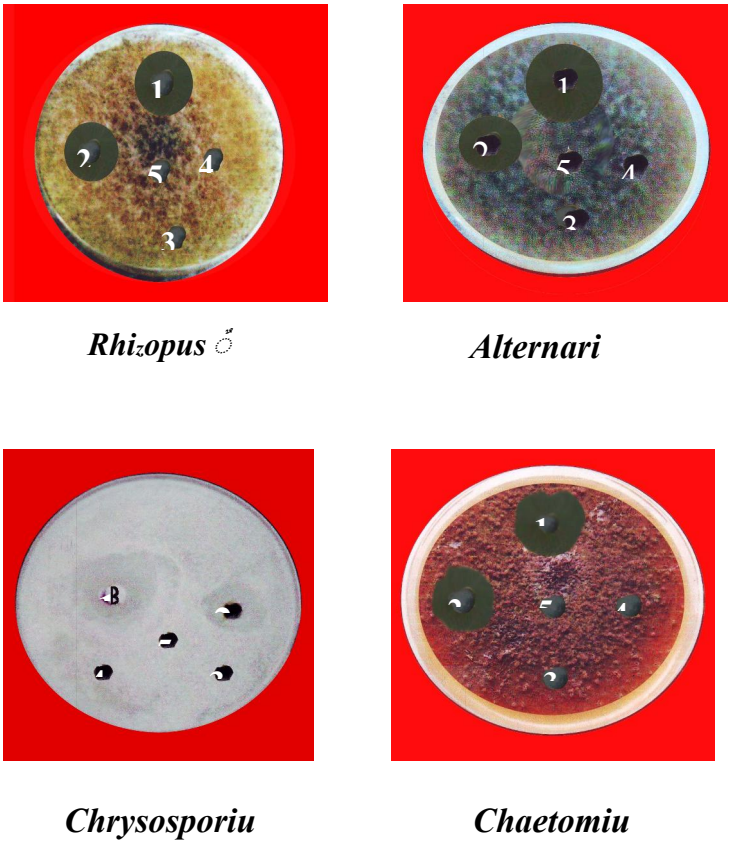
The highest zone of inhibition was 27 found against *Chrysosporium tropicum* followed by *B. polymyxa* may be this activity because this tow isolates can production Bacitracine, polymyxin, colistin, gatavalin, jolipeptid which considered peptide antibiotics cyclic there may be make unusual linkage with component of cell wall for fungi and prevent formation cell wall [15], while *B. subtilis* The highest zone of inhibition was 15 agains *Rhodotorula rubra*. In the case of *B. subtilis* some of these antibiotics have been found to be produced by a great variety of strains, e.g. subtilosin A, sublancin. Others are expressed strain-specifically, for example, ericin is produced by B. subtilis A1/3 only. Or may because surface of yeast smaller than filamentous fungi

therefore yeast sensitive while filamentous resistance [16].

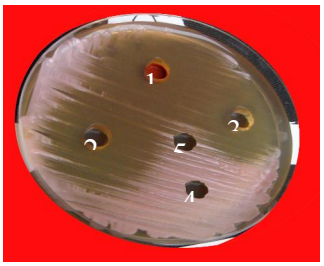
Either B. *licheniformis* didn't showed any activity against test fungal isolates may causes backed to referred [17] he reported that when glucose was added to culture media which seeded *B. licheniformis* results showed inhibited production of bacitracin synthesis which considered antifungal substance during first few hour of growth. he initially concluded that this delay in antibiotic production so may this causes to uncapability to inhibit test fungi.

Conclusion

The three bacterial isolates from soil were *Bacillus stearothermophilus, B.polymyxa, and B. subtilis* posessing appeared antifungal activity. From the above studies we had seen that the crude metabolites extract of these isolates were active against both pathogenic filamentous fungi and yeast therefore This study possible to be encouraging to treat fungal infections and it marks the beginning of extraction and purification of antibiotics produced by a species listed.



Plate(1):antifungal activity of four species Bacillus against filamentous fungi
1- Bacillus stearothermophilus
2- B.polymyxa
3-B.subtilis
4-B.licheniformis
5-Control



Rhodotorula rubra

Candida tropicalis

Cryptococcus neoformance

Candida albicans

Plate(2):antifungal activity of four species Bacillus against yeasts
1- Bacillus stearothermophilus
2- B.polymyxa
3-B.subtilis
4-B.licheniformis
5-Control

Table(1): Morphological and Biochemical characteristics of Bacillus

Test Species	Gram stianing	Spore formation	amylase	VP	citrate	Growth at 55°C	oxidase	catalase	Motility
Bacillus stearothermophilus	+	+	+	-	-	-	+	+	+
B.polymyxa	+	+	+	+	-	-	+	+	+
B.subtilis	+	+	+	+	+	-	+	+	+
B.licheniformis	+	+	+	+	+	+	+	+	+

Table (2) Antifungal activity of crude metabolites of four species Bacillus Diameter of inhibition zone (in mm)

Serious	Fungal isolates	Inhibition zone (mm)				
		Bacillus stearothermophilus	*B.polymyxa*	*B.subtilis*	*B.licheniformis*	control
1	*Rhodoturla rubra*	25	19	15	0	0
2	*Candida albicans*	13	12	12	0	0
3	*Candida tropicalis*	15	6	0	0	0
4	*Cryptococcus neoformans*	6	0	0	0	0
5	*Rhizopus stolonifer*	12	10	0	0	0
6	*Chrysosporiumtropicum*	27	20	0	0	0
7	*Alternaria alternate*	15	13	0	0	0
8	*Chaetomium globosum*	20	15	6	0	0

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