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A STUDY OF FUNGISTATIC ACTIVITY AGAINST *APHANOMYCES* SP. OF ACTIVE COMPOUNDS PRODUCED FROM *BACILLUS SUBTILIS*

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ABSTRACT

Bacillus subtilis isolated from Common carp fish intestine produces volatile compounds (VOCs), Bioassay in sealed dishes insure their ability to inhibit the growth and spore germination and mycelium growth of *Aphanomyces* sp. Totally 21 volatile compounds were extracted by solid-phase microextraction (SPME) followed by gas chromatography-mass spectrometry (GC/MS) to identify the VOCs.

KEYWORDS: *Bacillus subtilis*, *Aphanomyces* sp., gas chromatography-mass spectrometry (GC/MS), VOCs, Common carp.

INTRODUCTION

With the increased demand for fish as a source of protein, fish production in aquaculture was greatly increased during the last few decades to meet this task, this make fish health in aquaculture in danger du to the crowding, high density stocking of fish and accumulation of their own metabolic wastes in addition to the environmental factors, physical conditions all play apart in determining the susceptibility of fish to diseases[1], this means more usage for pesticides and antibiotic to control these diseases but these chemicals had its hazard effects on the environment and human, accordingly many countries tend to use biological control as a sustainable alternative method to chemical usage[2], from these methods using some bacterial strains such as which have the ability to produce antifungal compounds *Bradyrhizobium japonicum*, *Pseudomonas fluorescens*، *Bacillus* spp. and *Aeromona*s.[3,4,5] *Bacillus* spp. had wide range of applications such as biological degradation of minerals[6], inducers for plant growth[7], used in self-healing of cracks in concrete.[8] Many studies reported significant antifungal activity against *Pythium* sp., *Botrytis cinerea*, *Puccinia pelargonii-zonalis* and *Fusarium oxysporum*.[9,10,11] *Bacillus* spp. play a major role in

biological control according to their safety and potential considered high and have different mechanisms resulting in biological control which are competition to substrata, antagonism by antibiosis and production of antibiotics, degradation of cell wall by enzymes.[12,13,14]. The aim of this study to detect and identify Volatiles Compounds produced from nonpathogenic *B.subtilus* and to identify their antagonistic activity to reduce the growth and inhibit the spore germination of *Aphanomyces* sp. *in vitro.*

MATERIALS AND METHODS

Isolation and Identification of *Bacillus subtilis* from Fish. Twenty healthy fish of Common carp (*Cyprinus carpio*) were collected from local markets, with weight range (1000-1200) gm during October 2014 these fishs were transferred to the laboratory in aquariums and vivisectioned under sterile conditions by following the method[15] with few modifications, fish intestine transferred to a blinder and diluted with 5 ml of (1.5% Nacl) solution per one fish intestine this mixture transferred into bath water $80C^0$ for 20 min then cooled with tab water. The intestine mixture was spread in Petri dishes contain nutrient agar contain (1.5% Nacl), after that these petridishes incubated at $37C^0$ for 24h then the bacteria was purified and cultured in MS media and identified depending on the morphological and biochemical features.[17]

Isolation of the fungus *Aphanomyces* sp. This fungus was isolated randomly from infected common carp eggs depending on the method.[18] according to the morphological characteristics and spore releasing method depending on the classification key.[19]

Antagonism test *in Vitro* of (VOCs) produced from *B. subtilis* against *Aphanomyces* sp. This test has been done by separated sealed petridishes method mentioned in[20] with some modifications, these dishes separated with sterile slide first half (which inoculated with *B. subtilis*) contain modified MS media by adding (1.5% from both Agar agar and sucrose and 0.4% TSA (W/V)) according to the method followed by.[21] The second half contain Yeast extract agar, half number of these dishes inoculated with touch of the fungal colony and the remained number inoculated by spreading 100 µl of (10^6 spore/ml) which prepared following the method[22], to test the antagonistic activity against spore germination. Control Petri dishes do not inoculated with *B. subtilis*, this test done with three replicates. The dishes incubated at $28C^0$ for 72h, inhibition percentage calculated by Appling the formula in[23] which.

Inhibition percentage % = Diameter of fungal colony in control- diameter of fungal colony in test×100/ diameter of fungal colony in control.

Extraction of (VOCs) produced from *B. subtilis* Three Solid Phases Micro Extraction (SPME) fibers from SOPELCO were used (7µm polydimethylsiloxane (PDM), 65µm polydimethylsiloxane-divinylbenzene (PDMS/DVB), 50/30 µm stable flex divinylbenzene-carboxene-PDMS (CAR/DVB/PDMS)). *B. subtilis* was inoculated in 50 ml of MS media in 100 ml vial incubated at $28C^0$ for 72h, after that transferred to hot water $50C^0$ for 30 mins. The SPME fibers pierced into the parafilm and then exposed to the headspace of the vial sample for 45 mins, after that SPME fibers were directly inserted into the front inlet of gas chromatography.

GC/MS analysis SPME fibers were desorbed at 220°C for 1 min in the post injection GC-MS Qp2010(SHIMADZU) with an RTX-5MS column (30 m length, 0.25mm inside diameter, 0.25 µm), each run was performed for 25 min. Initial Temperature of the oven was 35 C0held for3 mins then ramped up in ratio(5C0. min-1) to 180C0, more ramped up at ratio (50 C0.min-1) to 250C0 and held to 3mins.VOCs identified by comparisons of their mass spectra with compounds with known spectra in database and confirmed by comparisons of their retention time with those in data published in literatures.[24,25]

RESULTS AND DISCUSSION

The VOCs produced by *B. subtilis* reduced mycelial growth and spore germination (Fig.1a and b) respectively, in comparative to control VOCs had reduced the fungal mycelium and fungal colonies germination significantly. The inhibition of *Aphanomyces* sp. was about 30% to 45% compared with control after 72h incubation, its clear that the VOCs unable to kill the fungus but had significant inhibitory effect on fungal mycelium growth. its clear that *B. subtilis.*

Produced more than one bioactive compound, authors[20] found VOCs produced from *B. amyloliquefaciens* NJN-6 can play important roles over short and long distances in the suppression of *Fusarium oxysporum,* other researchers[24] revealed that VOCs produced by *B. subtilis* had inhibitory effect on soil-borne fungi and effective control of overwinter seclerotia germination of *Seclerotinia seclerotorium.*

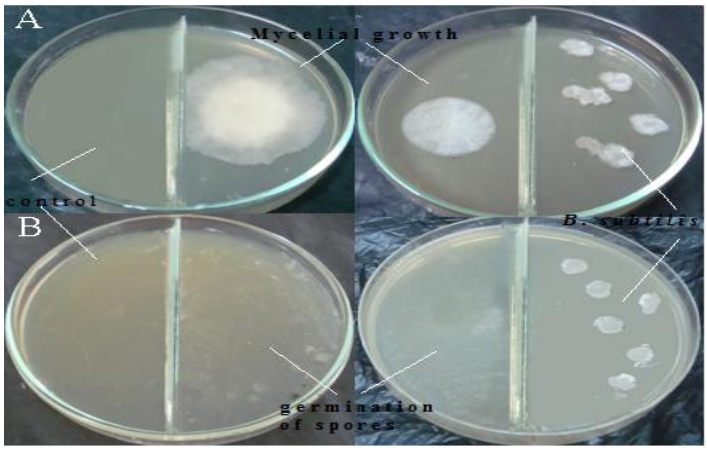

Figure1: antagonistic activity of *B. subtilis* against *Aphanomyces* sp. Analysis of VOCs from *B. subtilis* (A) Mycelial growth was inhibited in the presence of the bacteria cultured in the different compartments (right), compared to the control (left). (B) Volatile organic compounds produced by *B. subtilis* affected the germination of spores (right), compared to the control (left).

Eventually, 50/30 µm stable flex divinylbenzene-carboxene-PDMS (CAR/DVB/PDMS) was the best to extract VOCs because the most peaks areas and peaks numbers of compound (Fig.2). Totally 21 organic compound were determined by GC/MS method including eight ketone, six alcohol, three ester, one acid, one phenol, one amine and one heterocyclic (tab.1). The identified VOCs were purchased from Sigma-Aldrich and compared with the results of.[20,24] The VOCs are infochamicals because their ability to act over a wide range of distances and their spheres of activity extended from proximal interaction to greater distance via diffusion in air and water.[26]

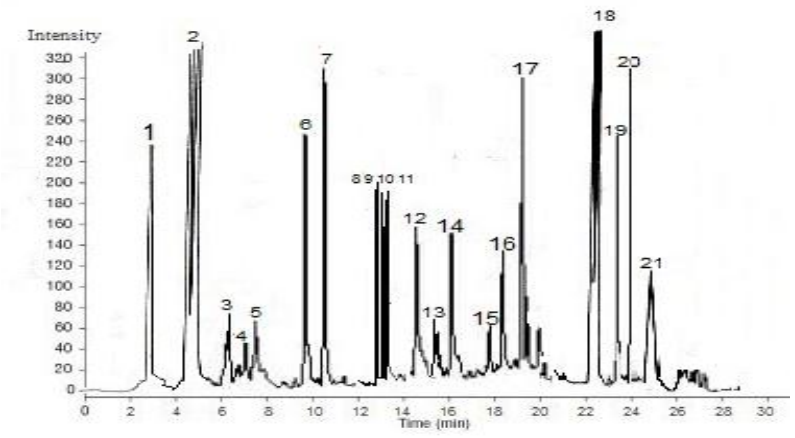

Figure 2: GC/MS profiles of *B. subtilis* VOCs, peaks of organic compounds numbered from 1 to 21.

Table 1: VOCs produced by *B. subtilis* determined by SPME-GC/MS method.

Peaks numbers	Retention Time (min)	Name of compound	Classification of compound
1	3.055	Acetic acid	Acid
2	4.711	Ethanone	Ketone
3	5.701	6-methyl-,2-Heptanone	Ketone
4	6.415	3-Octanone	Ketone
5	7.463	1-Hexanol,2-ethyl-	Alcohol
6	9.147	2-Nonanol	Alcohol
7	10.589	2-Decanone	Ketone
8	12.846	2-Undecanone	Ketone
9	12.942	2-Tridecanon	Alcohol
10	13.90	2-Dodecanone	Ketone
11	13.992	2-Dodecanone	Ketone
12	14.244	2-Dodecanol	Alcohol
13	15.862	1-Dodecanol	Alcohol
14	16.249	2-Tridecanon	Ketone
15	17.859	Diethyl Phthalate	Ester
16	18.553	Phenol,2,6-bis(1,1-dimethylethyl)-4-(1-methylpropyl)	Phenol
17	18.867	4-Ethyl lcyclohexanol	Alcohol
18	22.588	Morpholine, 4-octadecyl-	Heterocyclic
19	22.874	1-Hexadecanamine,N,N-dimethyl-	Amine
20	23.861	Dibutyl Phthalate	Ester
21	25.633	Methyl ester	Ester

VOCs produced by *B. subtilis* had been demonstrated to have antimicrobial activity, interacted with the host and behaved as a signal among bacterial communities. For example, 2-Decanal was shown to inhibit *Sclerotinia sclerotiorum*[24], Phenols had well established antiseptic uses in clinical applications for along time because of their toxic activity for cells.[27] Therefore, it was likely that the two Heterocyclic compounds played an important role in the antifungal effects of the VOCs this result agreed with.[20] The alcohol, ester compounds were produced at very low levels, bout they completely antagonized *F. oxysporum* at a concentration of 200µl *in vitro*[20]**.,** Considering both ketone contents and antifungal effects, 2-undecanone, 2-dodecanone and 2-tridecanone might be considered active ketone compounds. ketones were produced at high levels. Ketones like 2-decanone and 2-nonanone exhibited 100% inhibition, but their contents were low in VOCs, as indicated by GC/MS method.[20,24]

CONCLUSIONS

B. subtilis produce VOCs which have the potential *in vitro* antifungal activity against Oomycetes and our results are useful for the better understanding of the biological control mechanisms by *B. subtilis* against fungi in general.

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