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Antifungal Protein ~35kDa Produced by *Bacillus cereus* Inhibits the Growth of Some Molds and Yeasts

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An antifungal protein synthesized by *Bacillus cereus* has been partially purified by the use of ammonium sulfate precipitation and Sephadex-G-200 column chromatography. The protein was produced from *Bacillus cereus* grown in potato dextrose broth medium (PDB) at 30ºC for 3 days at 100 rpm. The protein showed antagonistic effect against some fungi and yeasts. Crude extract from medium and semi-purified protein were tested *in vitro* against both fungi and yeasts using the disc diffusion method in order to detect the inhibitory effect of the protein. Zones of inhibition of the following diameter were found (mm) were *Alternaria alternate*[28], *Rhodotorula glutinis*[20], *Fusarium sp.*[16], *Rhizopus sp.*[15], *Penicillium digitatum*[13], *Mucor sp.*[13] and *Aspergillus niger*[10]. The isolated protein was found to have a molecular weight of ~35kDa by sodium deodecyl sulfate-poly acrylamide gel electrophoresis. The data showed that the protein of *Bacillus cereus* has antifungal activity, a fact which points to the possibility of using it as a biocontrol agent against some fungi, findings which emphasize the potential role of *B. cereus* as an important biocontrol agent.

Key words: *Bacillus cereus,* Protein~35kDa, Molds, Yeasts.

Bacillus spp. can produce structurally diverse secondary metabolites which exhibit a broad spectrum of antibiotic activity[1,2]. Many of these antifungal substances have been characterized and identified as being peptide antibiotics[3]. Antifungal peptides produced by *Bacillus* species, include mycobacillins[4,5], iturins[6], bacillomycins[7,8], surfactins[9], mycosubtilins[8], fungistatins[10], subsporins[11] and rhizocticins[12]. *Bacillus* sp. also produces a range of other metabolites including chitinases and other cell-wall degrading enzymes[13,14] and some antifungal proteins[15,16].

The use of antagonist bacteria, or preparations which are derived from them, is an efficient way of suppressing soil micromycetes and phytopathogens which cause root-rot of cultivated plants. Members of the genus *Bacillus* show considerable promise as antagonists of fungal phytopathogens[17] and as a rule their inhibitory effect is primarily due to their ability to produce low-molecular-weight antibiotics, including antibacterial peptides[18].

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Bacillus cereus is particularly well-known as a producer of antibacterial antibiotics[19,20,21,2] since it produces zwittermicyne A, an aminopolyol antibiotic which is very effective in suppressing damping-off of alfalfa, caused by *Phytophthora medicaginis*[23]. A number of reports have demonstrated the biocontrol potential of *B. cereus* against several plant pathogens[3], but the involvement of antibiosis in the inhibition process has not been widely described. Here, we sought to isolate and characterize molecular-weight metabolites produced by *Bacillus cereus* in liquid culture which are active against various molds and yeasts which contaminate foods and act as phytopathogens.

MATERIALS AND METHODS

Bacteria and fungi used

Fifty isolates of bacteria and fungi were obtained from diverse sources including soil and vegetables. One bacterial isolate was found to be active against a variety of molds and yeasts and this was chosen for our study.

Tested organisms

The following molds, *Aspergillus niger*, *Alternaria alternate*, *Fusarium sp*., *Rhizopus sp*., *Mucor sp.* and three genera of yeasts, *Candida tropicalis*, *Rhodotorula glutinis* and *Geotrichum candidum* were used in this study; all were obtained from the Food Microbiology Laboratory, College of Food and Agricultural Sciences, King Saud University.

Bacterial identification

Identification of the isolates was determined based on sequence of 16S rRNA gene. The DNA genome of isolates was extracted according to the procedures of SciGenom Labs Pvt Ltd, Plot No. 43A, SDF 3rd Floor; CSEZ, Kakanad, Cochin, Kerala 682037.

Bacterial growth in shake flasks

Bacillus cereus was grown in shaking culture. A *Bacillus* colony (grown on potato dextrose agar) was inoculated into potato dextrose liquid medium (Oxoid) and incubated for 3 days at 30 °C with constant shaking (100 rpm).

Antagonistic effect of cell free supernatant of the *Bacillus cereus* isolate

The *Bacillus cereus* isolate was incubated for three days at 30°C with constant shaking (200 rpm). After 24 h, the resultant culture was centrifuged (5000 rpm) for 20 min. cell free supernatant was filtered through millipore filter (0.22 µm) and tested for the presence of antifungal activity. In order to achieve this, a filter paper disc (5mm diameter) was saturated with 100 µl of the cell free supernatant was placed in the center of a plate containing a culture of the fungus or yeast under test (both were inoculated onto the surface of PDA medium). The plates were incubated for 3-7 days at 30ºC, when the diameter of the inhibition zone around the disc was measured; three replicates were set up per fungus or yeast[24].

Purification of a 35 kDa

The method was used with some modifications, The *Bacillus sp.* was inoculated in 1 L of PDB medium and incubated at 30ºC in a rotary shaker (100 rpm) for 3 days. The culture was centrifuged at 5,000 rpm for 15 min and the cell-free supernatant was collected. The protein was precipitated overnight with 40% ammonium sulphate, by slowly stirring at 5ºC for 1 h. The precipitate was then dissolved in distilled water and dialyzed using a cellulose membrane against distilled water. The insoluble residues were removed by centrifugation at 12,000 rpm at 5 °C for 15 min. and the resultant soluble portion (with antifungal activity) was stored at 4°C for further purification[25].

SDS-PAGE of the separated components (the Laemmli procedure) was carried out in 12% polyacrylamide supplemented with 0.1% SDS[26]. The gel was stained with a 0.1% solution of Coumassie Brilliant Blue G-250 (Serva, Germany) (0.1% CBB R-250, 20% methanol, 0.5% acetic acid) and incubated at room temperature on an orbital agitator for at least one hour, The stain was the decanted and the gel was briefly rinsed with distaining solution (20% methanol: 10 glacial acetic acid: 70% distilled water) with continuous agitation until clear protein bands were obtained.

RESULTS AND DISCUSSION

Identification of chitinolytic isolates

Amplification of 16S rRNA gene resulted in a specific DNA fragment of approximately 1500 bp (Fig. 1). DNA sequence analysis of the PCR products using the BlastN revealed that the *Bacillus* isolate showed significant similarity with

Table 1. Antifungi activity of *Bacillus cereus* protein crude (cell free supernatant) and semipurified protein (precipitated by ammonium sulphate)

Molds and yeasts	Cell free supernatant medium	Semipurified protein
Rhodotorula sp	10	20
Alternaria alternata	15	28
Rhizopus sp	8	15
Mucor sp	6	13
Penicillium digitatum	6	13
Geotrichum candidum	-	-
Aspergillus niger	6	10
Candida tropicalis	-	-
Fusarium sp	8	16

Bacillus cereus based on nucleotide homology and phylogenetic analysis (Fig. 2).

Data in (Table 1) shows the diameter (mm) of inhibition zones of *Bacillus cereus* protein produced against the growth of different kind of molds and yeasts, with the width of the inhibition zone produced in dual culture tests showed the degree of antagonism against the tested organisms. The zone of inhibition (mm) for semi-purified protein solution and medium free bacterial cell inhibited (zone of inhibition, mm) *Alternaria alternate* (28, 15), *Rhodotorula glutinis* (20, 10) *Fusarium sp*. (16, 8), *Rhizopus sp*. (15, 8) *Mucor sp*. (13, 6), *Penicillium digitatum* (13, 5), *Aspergillus niger* (10, 6) respectively. The size of the inhibition zones between the *Bacillus cereus* and the tested microorganism was directly related to degree of inhibition of degree of inhibition against *B. cereus*. No inhibitory effect of *Bacillus cereus* protein was seen against *Geotrichum candidum* and *Candida tropicalis*. The mode of antagonism generally observed with *Bacillus cereus* is antibiosis[27]. This is supported by reports that most *Bacillus* sp. produce a number of antibiotics, such as bacillomycin, fengycin, mycosubtilin and zwittermicin, which are all effective at suppressing growth of target pathogens *in vitro* and/or *in situ*[28] and showed that *Bacillus cereus* exhibits antifungal activity against *Saccharomyces cerevisiae* with the largest inhibition zone produced measuring up to (16.25 mm) and also against *Candida albicans* (maximum growth inhibition zone, 31mm)[29].

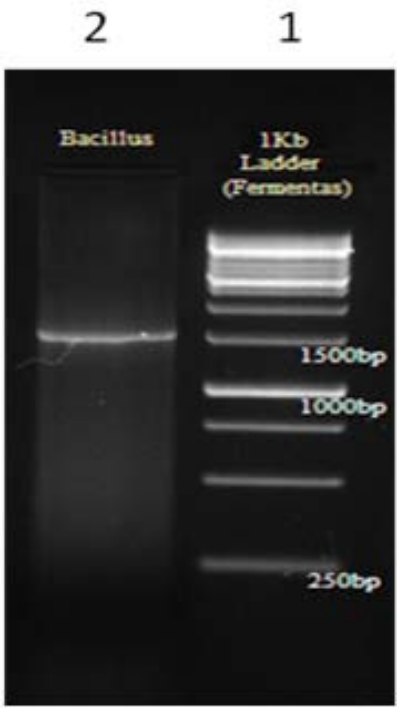

Fig. 1. Amplicon photograph (Amplicon was electrophoresed in a 1% Agarose gel and visualized under UV, lane 1: 1Kb ladder, lane 2: DNA fragment of the *Bacillus* isolate)

This results of this study show that a protein secreted by *Bacillus cereus* acts as an antibiotic. In SDS–PAGE, the protein appeared as a single protein band after staining the gel with CBB R-250, and the molecular mass was estimated of about 35 kDa (Fig.3)

Our results show that the protein of *Bacillus cereus* on SDS page strongly inhibited mycelial growth of a variety of different molds including *P. digitatum, Aspergillus niger, Mucor sp*. and the yeast *Rhodotorula glutinis* (Fig.4). The results also show that the antifungal activity of

Bacillus cereus protein exhibited a relatively wide spectrum of activity against plant pathogens. The antifungal protein isolated here has a native molecular mass of about 35 kDa; clearly more detailed biochemical and ultrastructural studies are now required to determine the primary target of the *Bacillus cereus* protein, especially since the antifungal component of *Bacillus cereus* may prove to be a novel protein.

B. cereus reported that X16, a halophilic strain isolated from salty soils, strongly inhibited the growth of *F. roseum* var. *sambucinum* on solid medium as well as on wounded potato tubers[14]. Our results do not concur with the findings who found antifungal activity in protein extracts of *B. cereus* that could pass through a 10 kDa filter, but was retained by a 3 kDa cut-off membrane filters, thereby indicating an apparent molecular mass between 3 and 10 kDa[22].

GCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCT
GCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATT
GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCG
GGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTA
AAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG
AGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCA
GTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTYCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATT
AAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTTGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACAACCCTAGAGATAGGG
CTTTTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATTTTAGTTGCCATCATTTAGTTGGGCACTTTAAGGTGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTAC
AAAGAGCTGCAAGACCGCGAGGTGGAGCTAATTTCATAAAACCGTTTTCAGTTCGGATTGTAGGCTGCAACTCGC
CTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTGGAGCCAGCC

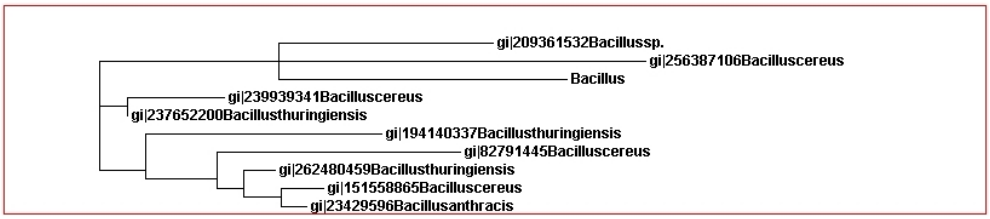

Fig. 2. Dendogram of the *Bacillus* isolate based on partial sequences of 16S rRNA gene (1415bp) with reference strains

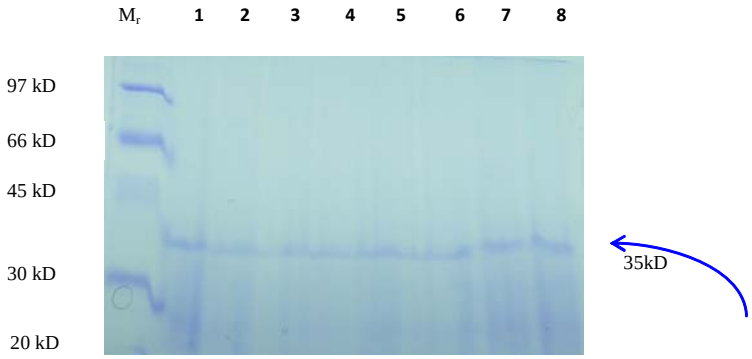

Fig. 3. SDS page analysis of *Bacillus cereus* protein secreted in PDA medium, precipitated by 80% ammonium sulphate and semi purified by cellulose membrane dialysis. M_r.: low molecular weight marker protein (Amersham, Bioscience, UK, 17044601), lanes (1-8); 50 µL of *Bacillus cereus* protein; the arrow indicates its location

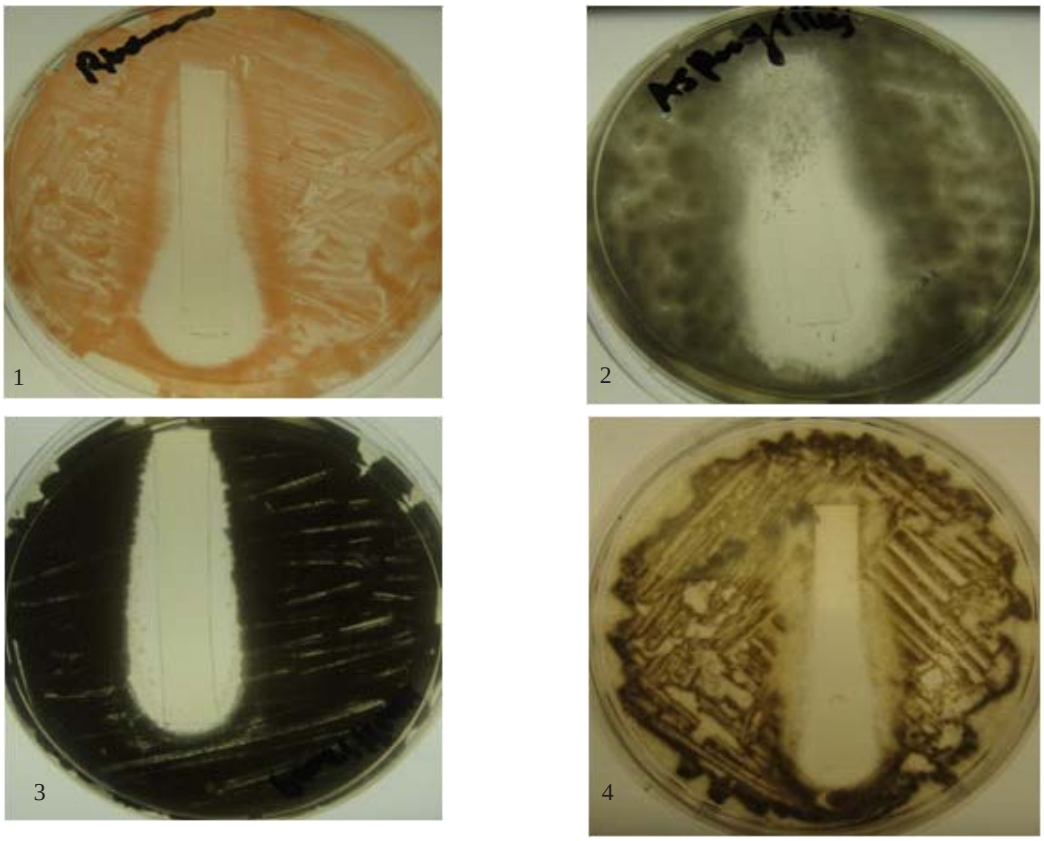

Fig. 4. Antifungal activities *in vitro* of the semipurified protein fractions (50 µL) each of on SDS page against 1. *Rhodotorula glutinis*, 2.*Aspergillus niger*, 3.*Penicillium digitatum* and 4.*Mucor sp*.

CONCLUSIONS

The evidence of this study shows the importance of *Bacillus cereus* protein secreted *in vitro* which has antagonistic action against some molds and yeast. Further studies, including, characterization tests, further purification, mass spectra and NMR and antifungal test against a wider range of fungi and yeast need to be conducted in order to confirm our findings; in summary, it is clear that *B. cereus* produces at least one compound which is capable of inhibiting the both mold and yeast growth.

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