

Molecular Detection of Antimicrobial Peptide Genes and Identification of Antifungal Compounds of *Bacillus* sp. against *Fusarium oxysporum* f. sp *cubense* Causing Wilt in Banana

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Abstract

Fusarium oxysporum f. sp *cubense* (Foc) is a serious infectious agent that causes *Fusarium* wilt of banana within tropical and semi-tropical regions of India. It is both necessary and critical to find an efficient **biocontrol** strategy to ensure banana production around the world. Twenty strains of *Bacillus* species from various banana rhizosphere regions were analysed using 16s rRNA and tested against *Foc* *in vitro*. Among **20 strains**, B18, B4 and B7 were observed to inhibit the mycelial growth of the pathogen. The *Bacillus* sp strains were analysed through PCR with specific primers for the presence of antimicrobial biosynthetic genes viz., Iturin A, Iturin C, Bacillomycin A, Surfactin and Bacillomycin D. The effective strain B18 was identified as *Bacillus cereus* based on biochemical and 16s rRNA region. In addition to antimicrobial peptide gens, the crude antibiotic extract from strain B18 was highly effective against *Foc* possibly owing to the presence of diverse antifungal compounds namely, 1- Dodecene, heptadecane, Pyrrolidine, Tetradecane, 1- Hexadecanol and Nonadecane, detected and identified using Gas chromatography and Mass spectrometry (GC/MS).

28 **Keywords:** *Bacillus*, *Fusarium oxysporum* f. sp *cubense*, antifungal compounds,
29 antimicrobial genes, GCMS

30 **Introduction**

31 Banana is commercially cultivated in many tropical and subtropical areas and remains to be
32 one among the world's most important fruit crops (Molina and Valmayor, 1999). India has
33 become the largest producer of bananas in the world with an annual production of 29.5
34 million tonnes from an area of 8.48 lakh ha (Indiastat, 2016). Among the diseases, *Fusarium*
35 wilt or Panama disease of banana (*Musa* spp.), caused by *Fusarium oxysporum* f. sp *cubense*
36 (E.F. Smith) "Snyder and Hansen", is the major restraint to banana cultivation and production
37 worldwide (Lin *et al.*, 2009). Application of fungicides and resistant cultivars are the only
38 management techniques available for controlling *Fusarium* wilt in banana. (Saravanan *et al.*,
39 2003; Geetha *et al.*, 2005). The loss of non-targeted valuable soil microorganisms due to
40 chemicals, have alerted researchers to look for alternative methods of management. *Bacillus*
41 species carries 24 varied Anti-Microbial Peptide (AMP) genes that allow the biosynthesis of
42 antibiotics like iturin, bacilysin, bacillomycin, fengycin, surfactin, mersacidin, ericin, subtilin,
43 subtilosin, and mycosubtilin (Chung *et al.*, 2008; Mora *et al.*, 2011; Rajesh kumar *et al.*,
44 2014). The generation of metabolites through bacterial cell culture with antimicrobial action
45 is a proven way to control plant pathogens. (Silo-Suh *et al.*, 2009). The detection of diverse
46 antifungal compounds of *Bacillus* species by GC-MS and their strong inhibitory effects
47 against pathogenic fungi (Dheepa *et al.*, 2016; Vinodkumar *et al.*, 2017). Therefore, *Bacillus*
48 species were considered effective in the production of antifungal compounds and
49 antimicrobial peptide genes against *Fusarium* wilt of banana. The objectives of the present
50 study include (i) Isolation and characterization of *Bacillus* spp from banana rhizosphere, (ii)
51 Detection of diverse antibiotic genes and (iii) Identification of antifungal compounds.

52 **Materials and Methods**

Isolation of Pathogen, *Fusarium oxysporum* f. sp. *cubense* (*Foc*)

Infected banana plants showing the typical wilt symptom were collected from different varieties of banana in Tamil Nadu. Corm portion was excised from the infected plant. The infected corm was cut into 1cm bits and surface sterilized with 0.5% sodium hypochlorite solution for 30 seconds and washed thrice in the series of sterile distilled water to remove the traces of sodium hypochlorite, and transferred to sterilized Petri plates containing potato dextrose agar (PDA) medium amended with 1000 ppm of streptomycin sulphate to avoid bacterial contamination (Burgess *et al.*, 1994). Then, the Petri plates were incubated at room temperature ($27\pm 2^{\circ}\text{C}$) for 7 days. The pathogen was identified through morphological and molecular characterization.

Isolation of *Bacillus* spp and screening against *Foc*.

Twenty strains of *Bacillus* spp., were isolated from the rhizosphere of banana. Antifungal activity of the *Bacillus* spp., was evaluated through a dual culture technique (Xiaoning *et al.*, 2014). Plates were incubated at $27 \pm 2^{\circ}\text{C}$. After 7 days of incubation, per cent inhibition (PI) of mycelial growth was calculated with the formula proposed by Dennis and Webster (1971).

PCR amplification of antibiotic genes

Bacterial genomic DNA was extracted (Knapp and Chandlee, 1996) and 16S rRNA interveining sequence specific BCF1 (5'CGGGAGGCAGCAGTAGGGAAT-3') and BCR2 (5'-CTCCCCAGGCGGAGTGCTTT-3') primer pair were used to confirm bacterial strain as *Bacillus* species described by Cano *et al.* (1994). These *Bacillus* species were also analysed for the presence of AMP genes with their respective primer pairs following procedures of Vinod Kumar *et al.*, 2017 and were described below for six genes.

Iturin A

The forward primer ITUD-1F (5'GATGCGATCTCCTTGGATGT3') and reverse primer ITUD-1R (5'ATCGTCATGTGCTGCTTGAG3') were used for amplification of iturin A gene

78 (647 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3 min, 35 cycles consisting of 94°C
79 for 1 min, 60°C for 30 s, 72°C for 1 min 45 s and 72°C for 6 min.

80 **Iturin C**

81 The forward primer ITUC-F (5' TTCACTTTTGATCTGGCGAT3') and reverse primer
82 ITUC-R (5' CGTCCGGTACATTTTCAC 3') were used for amplification of Iturin C gene
83 (506 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3 min, 40 cycles consisting of
84 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and 72°C for 10 min.

85 **Surfactin**

86 The forward primer SUR-3F (5'ACAGTATGGAGGCATGGTC3') and reverse primer SUR-
87 3R (5'TTCCGCCACTTTTTCAGTTT3') were used for amplification of surfactin gene (441
88 bp) (Vinod kumar *et al.*, 2017). PCR conditions: 94°C for 3 min, 40 cycles consisting of 94°C
89 for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 10 min.

90 **Bacillomycin A**

91 The forward primer BACA-F (5'TGAAACAAAGGCATATGCTC3') and reverse primer
92 BACA-R (5'AAAAATGCATCTGCCGTTCC3') were used for amplification of
93 Bacillomycin A gene (344 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3 min, 40
94 cycles consisting of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 10 min.

95 **Bacillomycin D**

96 The forward primer BACC-1F (5'GAAGGACACGGCAGAGAGTC3') and reverse primer
97 BACC-1R (5'CGCTGATGACTGTTCATGCT3') were used for amplification of
98 Bacillomycin D gene (875 bp) (Ramarathnam, 2007). PCR conditions: 94°C for 3 min, 35
99 cycles consisting of 94°C for 1 min, 62°C for 30 s, 72°C for 1 min 45 secs and 72°C for 6 min.

100 **Extraction and Bioassay of Crude metabolites against *Foc***

101 The isolates of three antagonistic bacteria (B18, B4, B7) were incubated at 28±2° C for 7
102 days. After incubation they were centrifuged at 10000 rpm for 10-15 min and the supernatant
103 was adjusted to pH 2.0 with concentrated HCl. The mixture was stirred at 100 rpm in an

orbital shaker for 8 hrs. After shaking, the precipitate was collected by centrifugation, re-suspended in 1mL of 1 M NaOH to adjust the pH 7.0. The resultant suspension was extracted twice with ethyl acetate. The ethyl acetate phase was transferred into the vacuum flash evaporator maintained at 60°C, at 80 rpm, till this fraction gets evaporated. The crude antibiotics was re-suspended in 1mL of methanol-chloroform mixture (1:1) and used for further bioassay and GC/MS analysis (Dheepa *et al.*, 2016). The bioassay was performed by Agar diffusion method (Hafidh *et al.*, 2011). The area of inhibition was also recorded.

Characterization of Crude Metabolites by GC-MS

Volatile antifungal and anti-bacterial compounds were identified by GC-MS using a column Elite-5MS (100% Dimethyl poly siloxane), 30 x 0.25 mm x 0.25 µm of equipped with GC Clarus 500 Perkin Elmer. The turbo Mass-Gold-Perkin-Elmer detector was used. The carrier gas flow rate was 1 mL/min, split 10:1, and injected volumes were 3 µL. The column temperature was maintained initially at 110°C at the rate of 10°C/min-No hold followed by increases up to 280°C at the rate of 5°C /min-9 min (hold). The injector temperature was 250°C and this temperature was held constant for 36 min. The electron impact energy was 70 eV, Julet line temperature was set at 2000°C and the source temperature was set at 200°C. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 aMU range. Using computer searches on the NIST Ver.2006 MS data library and comparing the spectrum obtained through GC/MS the compounds present in the crude sample were identified. Crude metabolites of the most effective strain of *B. cereus* B18 were analysed by GC-MS for the detection of active antifungal compounds responsible for the inhibition of *F. oxysporum* f. sp *cubense* (*Foc*) as proposed by Vinod kumar *et al.*, (2017).

Statistical Analysis

Data was analysed using IRRITSAT v. /93 software programme developed by International Rice Research Institute (IRRI), Philippines. Means differences of the treatment were evaluated with ANOVA and compared by Duncan's Multiple Range-Test (DMRT) (Gomez and Gomez, 1984).

Results and Discussion

The *Fusarium* wilt of banana pathogen produced white cottony mycelia growth with microconidia, macroconidia and chlamydospores 7 days after incubation at 27 ± 2°C. Based

on the morphological characters, the pathogen was confirmed as *Fusarium oxysporum* f. sp. *cubense*. In Addition, the *F. oxysporum* genus specific primers amplified a fragment of approximately 550 bp corresponding to the region of the 18S-23S rRNA intervening sequence for *F. oxysporum*. The amplified genomic product was partially sequenced at priority life sciences Pvt. Ltd., Bangalore, India. The rRNA homology searches were performed using the BLAST program through the internet server at the National Centre for Biotechnology Information, USA. The sequences analysis for isolates revealed that they had a nucleotide sequence identity of 98 - 100 % with the *F. oxysporum*. Since, the pathogen was isolated from banana, the identified pathogen was referred as *F. oxysporum* f. sp. *cubense*. The partial sequence of *F. oxysporum* f. sp. *cubense* isolates were submitted to the NCBI, Genbank, New York, USA. (MF576349). The molecular approaches are rapidly used for species level identification. Various techniques, including rDNA PCR (rDNA-intergenic spacer length polymorphisms) and 16S–23S intergenic transcribed spacer region have been used for identification and differentiation of bacterial species. The 16S rRNA gene sequence analysis has been described to be necessary mainly to detect some misidentification of *Bacillus* and related strains (Wu *et al.*, 2006). In present study, *Bacillus* spp., amplified at 546 bp corresponding region confirmed through 16s rRNA and the effective isolate B18 was sequenced and shows 100% similarity in NCBI database to be identified as *Bacillus cereus* (MH046059) (Fig. 1). Among twenty isolates of *Bacillus* spp., screened against *Foc* only three isolates were identified as effective. *B. cereus* strain B18 highly inhibited the growth of mycelium up to 58.89 % over control (Table 1). Further, 75 µl of crude extracts inhibited the mycelial growth of 407.00 mm² area of *Foc* under *in vitro*. *Bacillus* spp. produce a broad range of AMPs such as cyclic lipopeptides such as iturins, surfactins, fengycins, bacilysin, bacillomycin, mersacidin, and subtilin (Chung *et al.*, 2008; Rajesh Kumar *et al.*, 2014). Mora *et al.* (2011) reported that antimicrobial peptide (AMP) biosynthetic genes surfactin, bacilysin, fengycin,

159 bacillomycin, subtilin and iturin was examined in 184 isolates of *Bacillus* spp., from
160 rhizosphere, and non-rhizosphere regions of Mediterranean farm land area in Spain. Most
161 strains had between two and four AMP genes. Out of ten AMP genes were found from 55
162 *Bacillus* spp., only which *B. amyloliquefaciens* (VB7) highly inhibited the growth of *S.*
163 *sclerotiorum* (Vinodkumar *et al.*, 2017) that supported the finding of present investigation in
164 Banana. The AMP biosynthetic gene of iturin penetrates cell membranes and it leads to
165 cellular leakage (Ongena and Jacques, 2007). Zhang *et al.* (2013) reported that bacillomycin
166 produced by *B. cereus* disturbed the plasma membrane of *R. solani* abnormality in conidia
167 and mycelia were also observed. In our study, the 20 isolates of *Bacillus* spp, five AMP genes
168 associated to the antibiotics iturin A, iturin C, bacillomycin A, bacillomycin D, and surfactin
169 were prevalent. The effective *Bacillus* spp., with potential strains of *B. cereus* strain B18 and
170 B4, had five AMP genes responsible for the biosynthesis of antibiotic genes (Table 2).
171 *Bacillus* spp., are capable of producing an ample variety of secondary antifungal metabolites
172 that are varied in structure and function. Production of antifungal metabolites determines the
173 ability of *Bacillus* species to control plant pathogens. The crude metabolites of *B. cereus*
174 strains B18 were analysed by GC/MS to identify their production of antifungal compounds
175 (Fig. 2). Mainly vital antifungal compounds detected were, 1-dodecane, heptadecane,
176 pyrrolidine, 1-tetradecene and tetradecane (Table 3). Similarly, the results are also supported
177 by findings of Zhang *et al.*, 2011, which insist the crude lipopeptides extracted from cell-free
178 culture broth of *B. amyloliquefaciens* strain TF28 exhibited a wide-spectrum of antifungal
179 activity, but it strongly inhibited the growth and spore germination of *F. moniliforme*. Crude
180 extracts of *B. subtilis* have been reported to inhibit mycelial growth in *F. oxysporum* f. sp
181 *gerberae* (Suneeta *et al.*, 2016). Dodecanoic acid belonging to fatty acid with antibacterial
182 and antifungal activity (Kumar *et al.*, 2011). Too, Wang *et al.*, 2013 have identified 18
183 antifungal compounds produced by *B. amyloliquefaciens* W19 strain with high significant

antagonistic effect on *F. oxysporum* f. sp *cubense*. Possibly, the biocontrol by bacteria can be owing to the production of AMP genes, antifungal and antibacterial inhibitory compounds.

Conclusion

Management of *Fusarium* wilt of banana usually relies on several control methods. Hence, a study was conducted to understand the role and efficacy of antimicrobial genes and antifungal compounds from *Bacillus* spp., by extracting crude metabolites. GC/MS analysis of crude metabolite extract of the effective *Bacillus cereus* strain B18 revealed active antifungal compounds responsible for inhibition of *Foc*. Results showed five amplified AMP genes from all *Bacillus* spp., Therefore, the antifungal and antibacterial compounds identified through present investigation would help to control the *Foc* pathogen causing wilt in banana.

Consent & Ethical : NA

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270

S. No	Isolates	Radial growth of pathogen (mm)*	Percentage of inhibition
1.	B1	51.00 ^{gh}	43.33 (41.16) ^{ef}
2.	B2	64.00 ^b	28.89 (32.51) ^k
3.	B3	60.00 ^c	33.33 (35.26) ^j
4.	B4	40.00 ^k	55.56 (48.19) ^b
5.	B5	50.00 ^h	44.44 (41.81) ^e
6.	B6	58.00 ^{de}	35.56 (36.60) ^{hi}
7.	B7	42.00 ^j	53.33 (46.90) ^c
8.	B8	57.00 ^e	36.67 (37.26) ^h
9.	B9	47.00 ⁱ	47.78 (43.72) ^d
10.	B10	53.00 ^f	41.11 (39.87) ^g
11.	B11	48.00 ⁱ	46.67 (43.08) ^d
12.	B12	59.00 ^{cd}	34.44 (35.93) ^{ij}
13.	B13	51.00 ^{gh}	43.33 (41.16) ^{ef}
14.	B14	52.00 ^{fg}	42.22 (40.52) ^{fg}
15.	B15	47.00 ⁱ	47.78 (43.72) ^d
16.	B16	59.00 ^{cd}	34.44 (35.93) ^{ij}
17.	B17	69.00 ^a	23.33 (28.88) ^l
18.	B18	37.00 ^l	58.89 (50.12) ^a
19.	B19	63.00 ^b	30.00 (33.20) ^k
20.	B20	64.00 ^b	28.89 (32.51) ^k
21.	Control	90.00	0.00

271 Values are mean of three replications

272 Data in parentheses are arcsine transformed values

273 Means followed by a common letter are not significantly different at 5% level by DMRT

274 **Table 1. Efficacy of *Bacillus* spp against *Fusarium oxysporum* f. sp *cubense* under in**
275 **vitro**

276

S. No	Isolates	Bacillomycin D	Bacillomycin A	Iturin A	Iturin C	Surfactin
1	B1	+	+	+	+	-
2	B2	+	+	+	+	+
3	B3	-	-	+	-	+
4	B4	+	+	+	+	+
5	B5	+	+	+	+	+
6	B6	+	+	+	-	+
7	B7	+	+	+	+	+
8	B8	+	+	+	+	+
9	B9	+	-	+	-	+
10	B10	-	-	+	-	+
11	B11	+	-	-	-	+
12	B12	-	-	+	-	+
13	B13	+	+	+	-	+
14	B14	+	-	-	-	-
15	B15	-	-	-	-	+
16	B16	+	-	+	-	+
17	B17	+	+	+	+	+
18	B18	+	+	+	+	+
19	B19	+	+	+	+	+
20	B20	+	-	+	+	+

277 (+) = Positive (-) = Negative

278 **Table 2. Detection of antimicrobial biosynthetic genes among of *Bacillus* spp. through PCR**

S. No	Retention time (min)	Chemical name	Molecular formula
1.	2.55	1-Dodecene	C ₁₂ H ₂₄
2.	13.97	Heptadecane	C ₁₇ H ₃₆
3.	18.75	Pyrrolidine	C ₂₂ H ₄₁ NO ₂
4.	22.49	1-Tetradecene	C ₁₄ H ₂₈
5.	32.48	Tetradecane	C ₁₇ H ₃₆

Table 3. Antifungal compounds produced by *Bacillus cereus* strain B18 and identified by GC-MS.

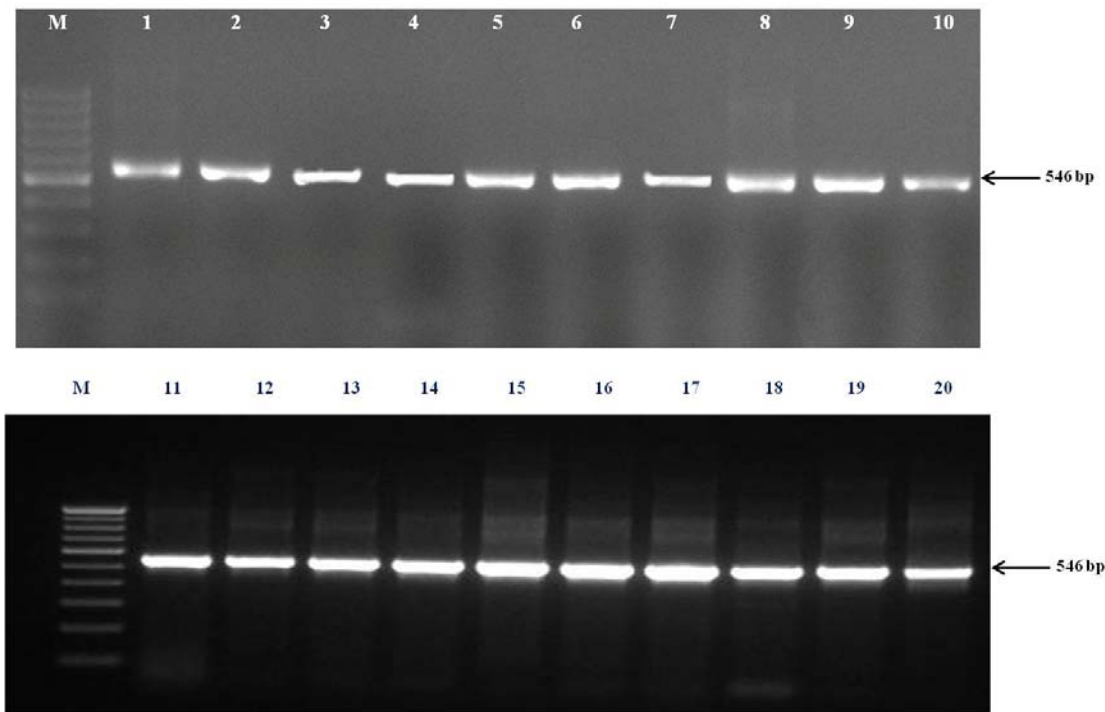
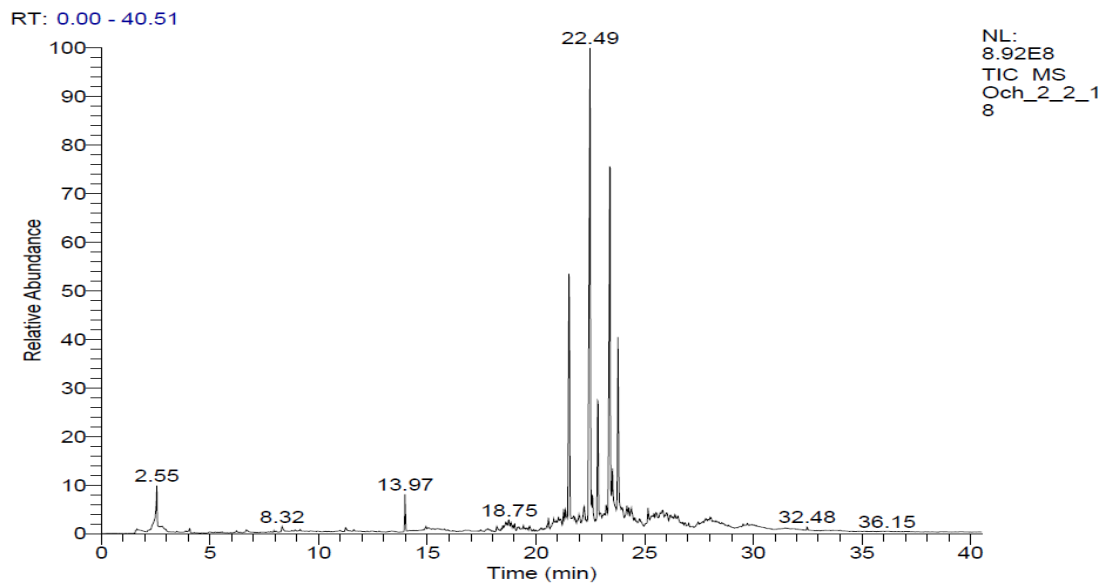


Fig. 1. PCR amplification of 16S-rRNA region of *Bacillus* isolates



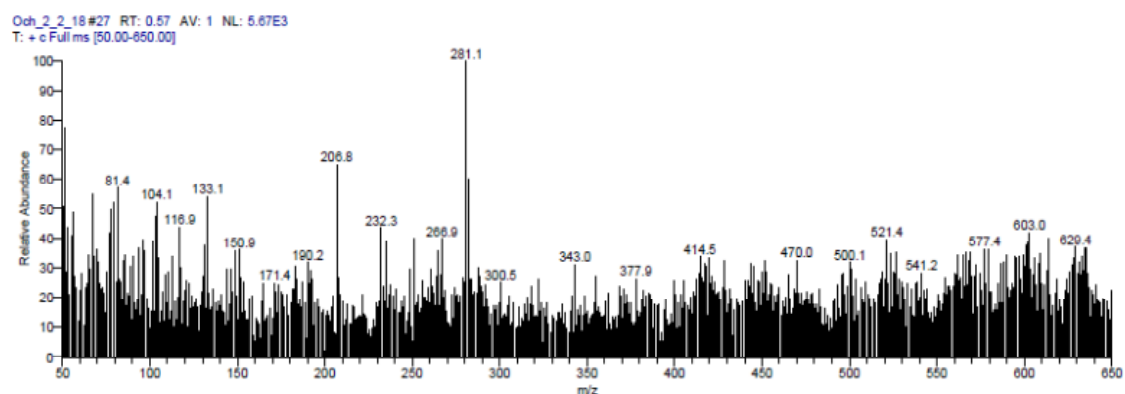


Fig. 2. Total Ion Chromatogram (TIC) and Mass Spectra (MS) of antifungal compounds identified from *B. cereus* strain B18 using GC/MS.