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Antifungal Trait of *Burkholderia gladioli* Strain VIMP02 (JQ811557)

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Abstract: ***Fungal pathogens affect many crops and can bring about severe changes in characteristics related to yield even at limited infection conditions. The objective of the present study was to investigate antifungal attributes of Burkholderia gladioli strain VIMP02 (JQ811557) by dual culture and agar well diffusion methods against Alternaria alternata, a soil borne pathogen of sugarcane and other crops. Culture filtrate and ethyl acetate extract obtained from culture supernatant showed prominent antifungal activity. Organic acids were detected by HPLC method. Acetic acid was predominantly produced by the culture under study. The GC-MS analysis of ethyl acetate extract revealed that antifungal fractions contained about 12 compounds including 9-nonadecene, 10-heneicosene, tetratetracontane and other fatty acid-, alcoholic- and phtalic acid derivatives. Presence of diverse metabolites supports the bacterium B. gladioli strain VIMP02 (JQ811557) to exploit it as antifungal agent.***

Keywords: Antifungal nature, *Burkholderia gladioli*, HPLC-, GC-MS analysis

1. Introduction

Phytopathogenic fungi are distributed worldwide and cause major as well as minor infections among wide varieties of plants, resulting in substantial losses. *Alternaria alternata* develops blight and leaf spots on large agricultural and horticultural crops such as cabbage, cucumber, broccoli, cauliflower, potato, beans, tomato and sugarcane [1]. In *Alternaria* leaf spot disease, brown necrotic spots are appeared on the leaf margin. With progression of disease the necrotic zone spreads towards the midrib which causes curling up and drying of leaves affecting yield [2]. Wide range of chemical fungicides is used to manage fungal diseases in crops which may cause environmental pollution and affect animal and human health. Besides this, fungal pathogens may develop resistance against these fungicides. Many researchers reported antifungal activities of their isolates including actinomycetes, yeast, bacteria as well as fungi [3, 4, 5, 6 and 7). There is a huge scope in developing novel fungicides for use in agriculture and food protection. In recent years there has been an escalating trend to assess the antimicrobial activity of medicinal plants and microorganisms due to resistance developed by phytopathogens. In the present study antifungal characteristics of *Burkholderia gladioli* strain VIMP02 (JQ811557) were investigated by using methods like dual culture, agar well diffusion and HPLC and GC-MS analyses.

2. Materials and Methods

2.1 Cultures

Bacterial culture employed for the present investigation was *B. gladioli* strain VIMP02 (JQ811557), an isolate by Mahamuni and Patil from sugarcane rhizosphere which had plant growth promoting attributes such as phosphate solubilization and composting ability [8]. Pathogenic culture *A. alternata* was obtained from the Plant Pathology Section of Vasantdada Sugar Institute, Manjari Bk., Pune.

2.2 Chitinase test

Culture of *B. gladioli* strain VIMP02 (JQ811557) was spot inoculated on colloidal chitin agar and incubated at 30^0C(± 0.2^0C) for 5 days and chitinase activity was examined as positive if there was zone of clearance around the colony [9].

2.3 Protease test

For protease test, culture was spot inoculated on milk agar having pH 7(± 0.2^0C) and incubated at 30^0C (± 0.2^0C) for 48 hrs. The development of clear zone around the colony against opaque background was considered as protease positive [10].

2.4 Cellulase test

The culture was spot inoculated on cellulose agar containing cellulose as sole carbon source and pH 7.5 and incubated at 30^0C for 48 hrs. The isolates showing growth on cellulose agar were considered cellulase positive. After incubation, plates having colonies were repeatedly treated with 0.5 per cent Congo red with intermittent washing by 1M NaCl solution. Cellulase producers develop clear zone around [11].

2.5 Organic acids

Pikovskaya's broth [12] was used to cultivate *B. gladioli* strain VIMP02 (JQ811557) at 30^0C for four days. High performance liquid chromatography (HPLC) was used to detect organic acids in broth. The culture broth was filtered through 0.2µm filter (Millipore) and 20µl of filtrate was injected to HPLC (Model- Waters Alliance Company) equipped with a UV detector. Organic acid separation was carried out on organic acid (Prevail) column (Make Grace) with specifications such as length 150cm and internal diameter (i.d.) 4.6mm and 25mM KH_2PO_4 as mobile phase. Retention time of each signal was recorded at a wavelength of 210nm.

2.6 Dual culture *in vitro* assay method

Antagonistic attribute of *B. gladioli* strain VIMP02 (JQ811557) was tested by dual culture *in vitro* assay method [13, 7]. The VIMP strain was spot inoculated at one end of the potato dextrose agar (PDA). After two days incubation at room temperature, 6mm agar disc using growth of fungal pathogen from fresh PDA agar culture, was placed at the other marginal side of the plate and incubated at room temperature for seven days. The radii of the fungal colony towards and away from the bacterial colony were noted to calculate per cent growth inhibition by formula,

Per cent inhibition = (R-r / R) × 100

Where "r" is the radius of the fungal colony opposite the bacterial colony and 'R' is the maximum radius of the fungal colony away from the bacterial colony.

2.7 Antifungal activity of culture filtrate and ethyl acetate extract

The culture grown in Pikovskaya's broth for seven days at 30^0C was centrifuged at 3000rpm for 10min and supernatant sterilized by passing it through millipore membrane filter (0.45µm pore size). The sporulated culture of *A. alternata* was inoculated into sterile molten PDA medium (45°C) and poured into sterile Petri dishes. Antagonistic activity of culture filtrate was detected by agar well diffusion technique.

Antifungal principles from the cell free filtrate were extracted by solvent ethyl acetate. Ethyl acetate extract was evaporated at room temperature and concentrated. About 500ml ethyl acetate extract was reduced to 15ml. Anti *A. alternata* activity of concentrated ethyl acetate extract was detected qualitatively by agar well technique using 100µl of the extract.

2.8 GC-MS analysis of ethyl acetate extract

The GC-MS analysis was conducted as explained by many researchers [14] with slight differences in analytical conditions and equipment make. Analysis was conducted using thermo gas chromatography coupled with ITQ 1100 mass detector and X-Caliber software and NIST Spectral data (GCMSMS, Thermo Fisher Scientific) with DB-5 MS capillary column (30 × 0.25µm internal diameter and 0.25µm film thickness). Analytical chromatographic conditions were as follows: 2µl sample injected; carrier gas helium @ 1ml per min; injector and transfer line temperatures used were 250^0C and 280^0C respectively; over temperature program included- 60^0C - 2min hold, 15^0C per min rate, increased to reach 160^0C remained at temperature for 0 min hold, 3^0C per min rate, increased to reach 200^0C remained at temperature for 1min hold, 2^0C per min rate, increased to reach 230^0C remained at temp for 1 min hold, 8^0C per min rate, increased to reach 285°C remained at temp for 6 min hold; split ratio = 1:50; ionization energy 70eV; mass range 50-650. The composition was determined by comparing peak retention times with those of reference standards.

3. Results

B. gladioli strain VIMP02 (JQ811557) showed protease and chitinase activities by inducing clear zones around the colonies on milk agar and colloidal chitin agar, respectively while cellulase activity was found to be negative. Organic acids produced by the culture in Pikovskaya's broth and identified by high performance liquid chromatography (HPLC) are presented in a **table 1** and peaks are represented in **figure 1**. In the aqueous culture filtrate of *B. gladioli* strain VIMP02 (JQ811557), four organic acids were detected. The highest amount of organic acid produced by strain VIMP 02 was acetic acid (80.91 mg/ 100 ml) and it was followed by the formic acid (3.71 mg/ 100 ml), oxalic acid (0.70 mg / 100ml) and citric acid (0.44 mg / 100ml) **(table 1)**. Approximate distribution of four organic acids (in per cent) produced in culture filtrate are presented in the pie diagram **(figure 2).**

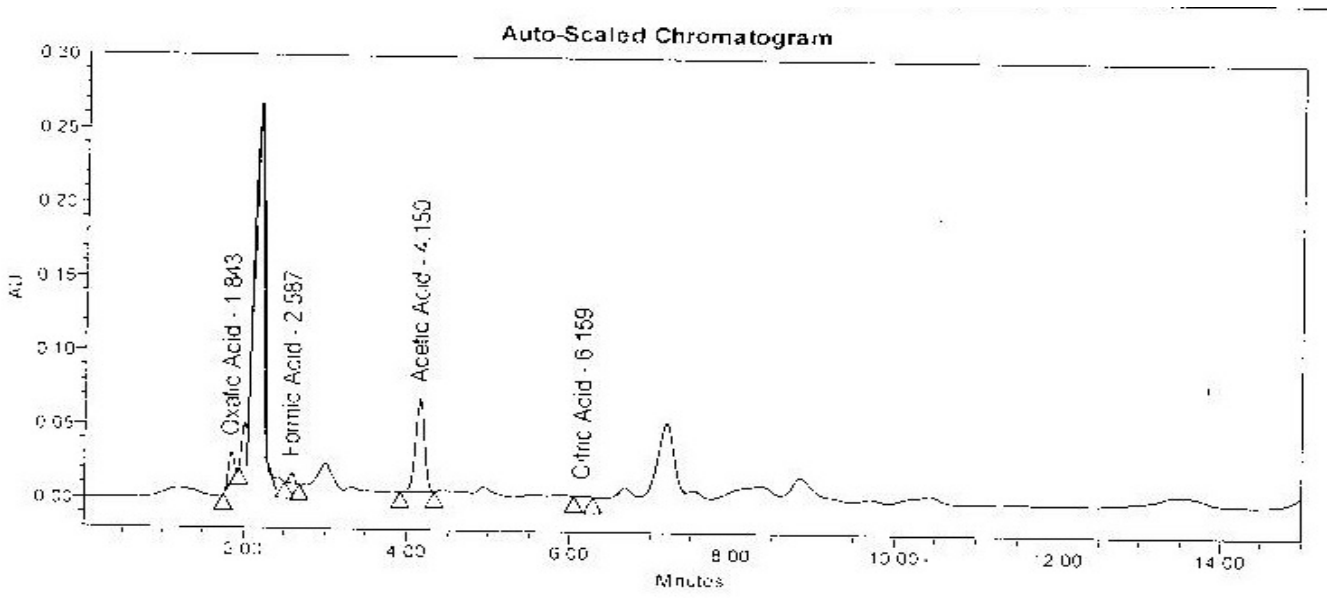

Figure 1: Chromatogram (HPLC) of organic acids from *B. gladioli* strain VIMP02 (JQ811557)

Table 1: Organic acid profile by HPLC

Sr. no.	Name of organic acid	Retention Time (Min)	Organic acid (mg /100 ml)
1	Oxalic acid	1.843	0.7
2	Formic acid	2.587	3.71
3	Pyruvic acid	-	-
4	Lactic acid	-	-
5	Citric acid	6.159	0.44
6	Gibberelic acid	-	-
7	Acetic acid	4.15	80.91

Note: "-" Not detected

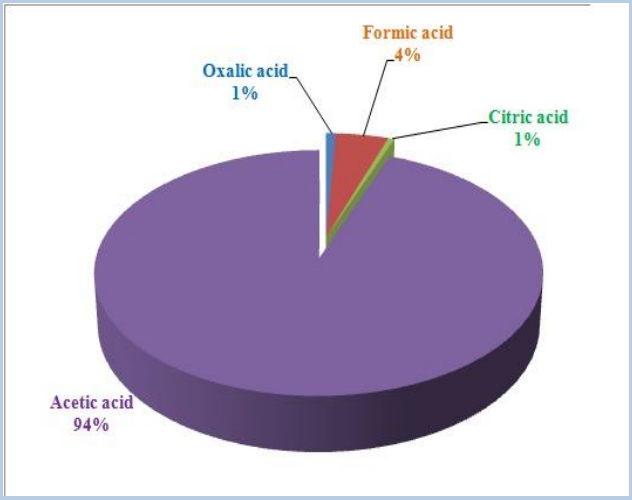

Figure 2: Organic acid content in culture filtrate (%)

Antagonist activity of *B. gladioli* strain VIMP02 (JQ811557) was evaluated against specific sugarcane pathogen *A. alternata* using dual culture technique. It revealed that the growth of *A. alternata* was inhibited with 56 per cent inhibition. Qualitative antifungal activities of aqueous culture filtrate and ethyl acetate extract from culture supernatant were determined **(figure 3)**. Both extracts inhibited the growth of *A. alternata* prominently by showing zones of inhibition (ZOI) 36mm (Standard deviation (SD) ±1.15 and 16mm (SD ±0.57), respectively.

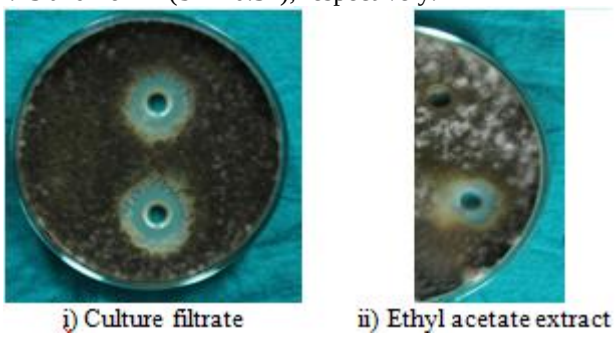

Figure 3: Antifungal activity

The GC-MS chromatogram of ethyl acetate extract obtained from *B. gladioli* strain VIMP02 (JQ811557) culture filtrate is presented in **figure 4**. The potential antifungal compounds identified by comparing peak retention times with those of reference standards from NIST Library data, are presented in **table 2** along with retention time (RT) in minutes, molecular formula and molecular weight (M.W.). It revealed the presence of 12 compounds that might contribute the antagonist trait of the *B. gladioli* strain VIMP02 (JQ811557). The first compound identified with less RT (12.20min.) was 9-Nonadecene, whereas the last compound which took longest RT (44.12min.) to identify was 4-phosphonobutanoic acid trimethylsiliester. **Table 2** revealed that tetratetracontane is a compound having highest molecular weight (M.W. 618) while lowest molecular weight is of Z-10-pentadece-1-ol (M. W. 226)

Table 2: GC-MS profile of ethyl acetate extract

Sr. No.	Name of compound	Molecular formula	M.W.	Retention time (min)
1	9-Nonadecene	$C_{19}H_{38}$	266	12.2
2	Z-10-pentadece-1-ol	$C_{15}H_{30}O$	226	16.01
3	7,9-Di-ter-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	19.35
4	Phthalic acid butyl 2-pentyl ester	$C_{17}H_{24}O_4$	292	20.31
5	10-Heneicosene	$C_{21}H_{42}$	294	20.82
6	Ethanol 2-(octadecyloxy)	$C_{20}H_{42}O_2$	314	20.98
7	Heptasiloxane hexadecamethyl	$C_{16}H_{48}O_6Si_7$	532	22.34
8	1,3,5,7,9-pentaethylbicyclo (5,3,1) pentasiloxane	$C_{10}H_{28}O_6Si_5$	384	24.66
9	Cyclohexane 1,1-dodecylidenebis(4-methyl)	$C_{26}H_{50}$	362	26.57
10	Tetratetracontane	$C_{44}H_{90}$	618	26.75
11	2-Phosphonobutanoic acid trimethylsiliester	$C_{13}H_{33}O_5PSi_3$	384	41.65
12	4-Phosphonobutanoic acid trimethylsiliester	$C_{13}H_{33}O_5PSi_3$	384	44.12

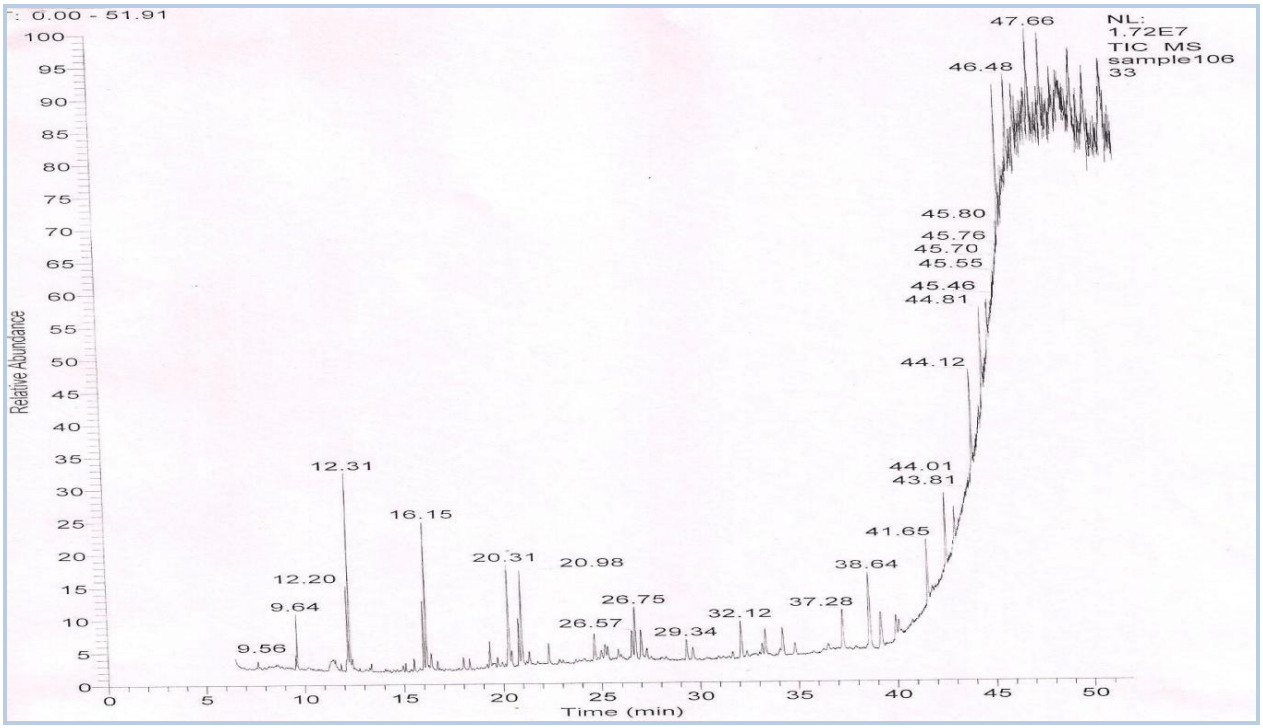

Figure 4: Gas chromatogram of ethyl acetate extract from *Burkholderia gladioli* strain VIMP02 (JQ811557)

Other high molecular weight compounds detected were heptasiloxane hexadecamethyl (M. W. 532), 2-phosphono-butanoic acid trimethylsiliester (M.W. 384), 4-phosphonobutanoic acid trimethylsiliester (M.W. 384) and 1,3,5,7,9 pentaethyl bicyclo (5,3,1) pentasiloxane (M.W. 384). In addition to these, other active principles including pthalic acid, phenol and alcohol derivatives were detected in the ethyl acetate extract under study. GC-MS profile of few compounds are presented in **figure 5–figure 10.**

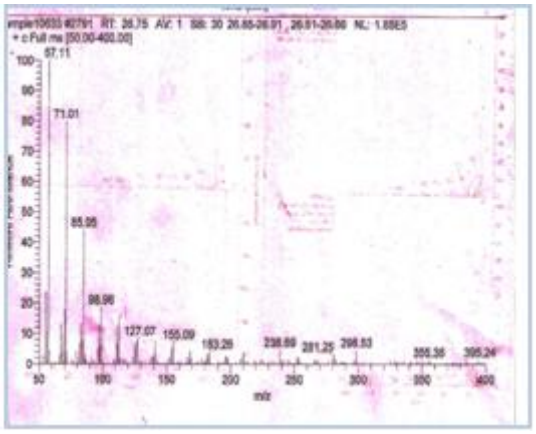

Figure 5: GC-MS profile of tetratetracontane at 26.75min

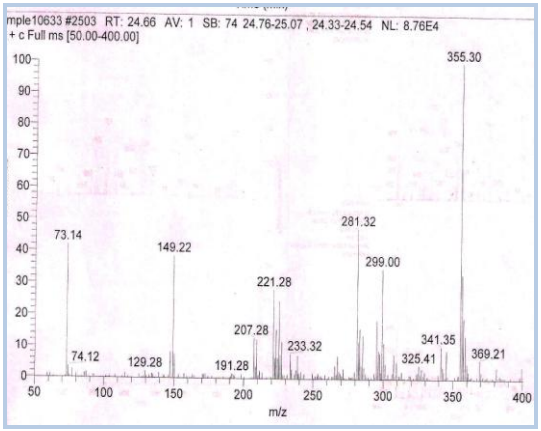

Figure 7: GC-MS profile of 1,3,5,7,9-pentaethylbicyclo (5,3,1) pentasiloxane at 24.66min

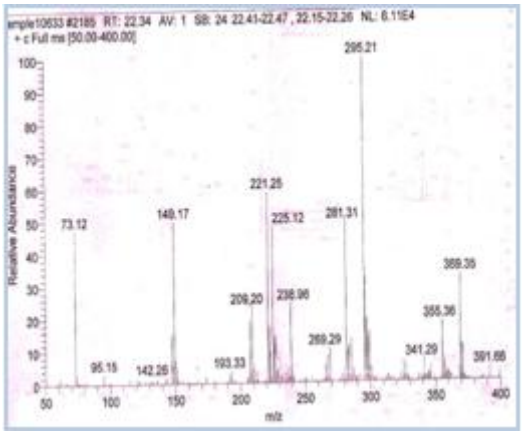

Figure 6: GC-MS profile of heptasiloxane hexadecamethyl at 22.34min

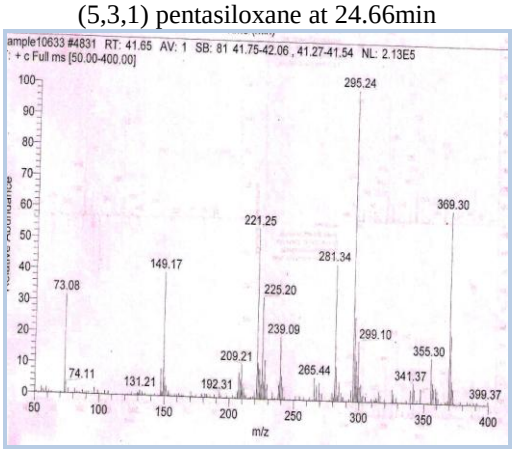

Figure 8: GC-MS profile of 2-phosphonobutanoic acid trimethylsiliester at 41.65min

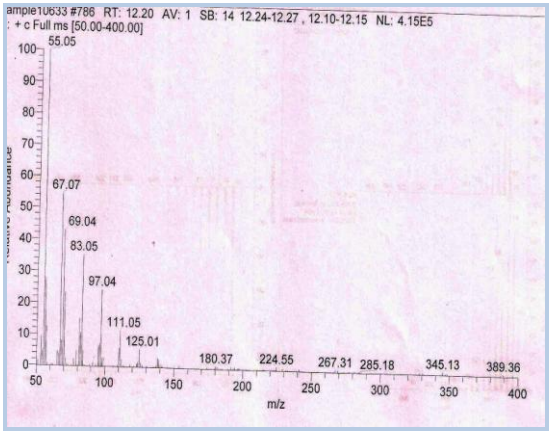

Figure 9: GC-MS profile of 9-nonadecene at 12.2min

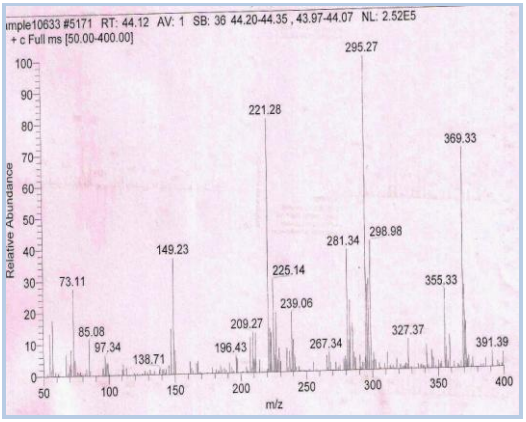

Figure 10: GC-MS profile of 4-phosphonobutanoic acid trimethylsiliester at 44.12min

4. Discussion

As indicated by large ZOI on agar plate, the bacterial culture filtrate exhibited more antifungal activity against *A. alternata*. It may be due to production of water soluble antifungal metabolites that diffused into the medium. These antifungal metabolites may intermingle with susceptible fungal cells linking cell wall damage [15]. Gerez *et al.* and Lavermicocca *et al.*, reported antifungal nature of organic acids like lactic acid, phenyl lactic acid, palmitic acid and acetic acid [16, 17, 18]. Culture under study produced variety of organic acids as presented in **table 1**. As elucidated positive chitinase and protease activities by *B. gladioli* strain VIMP02 (JQ811557), culture filtrate extract may also contained these cell wall degrading chitinolytic and proteolytic enzymes. This was the reason, which amplified antifungal properties of the *Burkholderia* culture filtrate under study. In addition, ethyl acetate extract revealed twelve compounds. Among these pthalic acid derivatives and nonadecene were reported as antioxidants and antimicrobials [19], while heneicosane, a fatty acid derivative was reported as antibacterial [20]. Tetratetracontane had antifungal activity [15]. Many compounds presented in **table 2** were similar to compounds reported as antimicrobials by earlier researchers; and also the presence of other residual compounds may be augmented antifungal potential as well as accelerated antifungal behavior of the *B. gladioli* strain VIMP02 (JQ811557). Results of the present investigation are in harmony with few metabolites reported both in culture filtrate as well as in ethyl acetate extract in earlier studies [15, 20, 19 and 21]. However, GC-MS analyses carried out in the present study and in earlier studies to be had great dissimilarity in revealed list of chemical compounds; this can be explained on the ground of differences in source or culture, cultural conditions and solvents employed for extraction.

5. Conclusion

The results of present investigation drew attention to antifungal principles present in culture filtrate and ethyl acetate extract from *B. gladioli* strain VIMP02 (JQ811557) culture supernatant. This was the first report of this bacterial culture stating its antifungal attribute especially against *A. alternata*. In future its impact on other fungal pathogens may be studied and field studies may be conducted to evaluate role of *B. gladioli* strain VIMP02 (JQ811557) in decreasing incidence of *Alternaria* related diseases.

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