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ANTIFUNGAL ASPECTS OF *BURKHOLDERIA* SP.VIMP04 (JQ867373) INHIBITING SOIL-BORNE FUNGAL PATHOGEN OF SUGARCANE

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ABSTRACT

The objective of the present study was to investigate antifungal features of *Burkholderia* sp.VIMP04 (JQ867373) by dual culture and agar well diffusion methods against *Ceratocystis paradoxa*, the causative agent of pineapple disease of sugarcane. Culture filtrate and ethyl acetate extract obtained from culture supernatant of *Burkholderia* sp.VIMP04 (JQ867373) showed prominent antifungal activity. HPLC analysis confirmed that bacterium produced different organic acids including acetic, oxalic and formic acids. Acetic acid was predominantly produced by the bacterium. The GC-MS analysis of ethyl acetate extract revealed that antifungal fraction contained 09 principal compounds including tetratetracontane, 10-Heneicosene, eicosene 7-hexyl, cyclohexane (6-cyclopentyl-3-(3-cyclopentylpropyl) hexyl, heptadecane 9-hexyl and other fatty acid-, alcoholic- and phtalic acid derivatives. Hence the culture under study can be used as biocontrol agent.

KEYWORDS: *Burkholderia*, Antifungal, HPLC, GCMS.

INTRODUCTION

The fungal diseases of sugar crops such as sugarcane and sugar beet are mainly caused by *Alternaria alternata, Rhizoctonia solani, Macrophomina phaeseolina, Fusarium oxysporum Aphanomyces chochlioides, Sclerotium rolffsi, Cercospora beticola and Ceratocystis paradoxa.* The phytopathogenic *C. paradoxa* is a causative agent of black rot or pineapple disease of sugarcane. The *C. paradoxa* affects parenchyma tissues of sugarcane internode after its entry through cut ends and develops brown black cavities. It deteriorates juice quality by decreasing the levels of sucrose and glucose (Patil and Jadhav, 1995).

Many researchers have documented the use of fungi and bacteria as biocontrol mediators such as *Trichoderma hamatum, Trichoderma viridae, Candida steatolytica, Pseudomonas fluorescens, Burkholderia, Bacillus* and others (Martin, 2003; El-Mehalwy, 2004; Kaur *et al.*, 2007; Zegeye *et al.*, 2011). *Burkholderia* species inhibited the growth of *Ustilago* and *Fusarium* (Antwerpen *et al.*, 2002). Gohar *et al.* (2010) characterized antibacterial agents produced by marine *Burkholderia cepacia.* Elshafie *et al.* (2012) reported antifungal activities of *Burkholderia gladioli pv. agaricicola*. The *Burkholderia tropica* was reported as growth inhibitor of fungi such as *Cholletotrichum gloesporioides, F. cumorum, F. oxysporum* and *Sclerotium rolffsi* (Tenorio-Salgado *et al.*, 2013). Most of the *Burkholderia* species suppress plant diseases by producing antibiotics, siderophores, organic acids, biocidal volatile organic components (VOC), phenolic- and phthalate- derivatives and enzymes including chitinase, cellulase, protease, etc. Bioactive components were well documented after agar well diffusion, high performance liquid chromatography (HPLC), gas chromatography and mass spectroscopy (GC-MS) studies by number of investigators in extracts obtained from different plants as well as bacterial and fungal cultures (Otun *et.al*, 2015; Usha *et.al*, 2015; Awla *et.al*, 2016). In the present study antifungal characterization of *Burkholderia* sp.strain VIMP04 (JQ867373) was carried out by dual culture, agar well diffusion, HPLC and GC-MS methods.

MATERIALS AND METHODS

Bacterial culture

Bacterial culture used under present investigation was *Burkholderia* sp.VIMP04 (JQ867373), the isolate by Mahamuni, and Patil from sugar beet rhizosphere (Mahamuni 2012; Mahamuni and Patil, 2012).

Phytopathogenic culture

The phytopathogenic culture of *C. paradoxa* was obtained from the Plant Pathology Section of Vasantdada Sugar Institute, Manjari Bk., Pune.

Culture media
Pikovskaya's broth and agar (Pikovskaya, 1948) media were used to cultivate *Burkholderia* sp.VIMP04 (JQ867373) at 30^{0}C for 04 days.

Potato dextrose agar (PDA) was used to cultivate phytopathogenic culture of *C. paradoxa* and also to check in vitro antagonistic activity at room temperature for 04 to 07 days.

Enzyme activities
Chitinase (Shanmugaiah *et al.*, 2008), *Cellulase* (Patagudi *et al.*, 2014) and *Protease* (Laxmi *et al.*, 2014) activities were performed by standard protocols.

HPLC analysis for organic acids
Organic acids were detected by high performance liquid chromatography (HPLC) in Pikovskaya's broth in which *Burkholderia* sp.VIMP04 (JQ867373) was cultivated at 30^{0}C (± 0.2^{0}C) for 04 days. 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. HPLC was operated withorganic acid (Prevail) column (Make Grace) having 150cm length, internal diameter (I.D.) 4.6mm and 25mM KH_2PO_4 as mobile phase at wavelength 210nm.

Dual culture method
Primary antifungal activity of *Burkholderia* sp.VIMP04 (JQ867373) was checked by the dual culture *in vitro* assay method (Pan and Jash, 2010). The culture was spot inoculated at one end of the PDA. After two days incubation at room temperature, 6mm agar disc using growth of fungal pathogen *C. paradoxa* 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,

$$\text{Per cent inhibition} = (A - a)/A \times 100$$

Where, "a" is the radius of the fungal colony opposite the bacterial colony and 'A' is the maximum radius of the fungal colony away from the bacterial colony.

Antifungal activity of culture filtrate and ethyl acetate extract
The culture *Burkholderia* sp.VIMP04 (JQ867373) grown in Pikovskaya's broth for 04 days at 30^{0}C (± 0.2^{0}C) was centrifuged at 3000rpm for 10min and supernatant sterilized by passing it through millipore membrane filter (0.45µm pore size). The sporulated culture of *C. paradoxa* was inoculated into sterile molten PDA medium (45°C) and poured into sterile Petri dishes. Antagonistic activity employing 100µl 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. Using 100µl of the ethyl acetate concentrate, antifungalactivity was detected qualitatively by agar well technique.

GC-MS analysis of ethyl acetate extract
The GC-MS analysis was done with thermo gas chromatography coupled with ITQ 1100 mass detector and X-Caliber software and NIST Spectral data (GCMSMS, Thermo Fisher Scientific). A DB-5 MS capillary column having 30 × 0.25mm internal diameter (ID) and coated with 0.25µm film thickness was injected with 2µl sample. The carrier gas helium (99.99%) was used at flow rate of 1ml per min. in split mode (1:50). The temperature of the column was programmed at 60^{0}C to 280^{0}C. The injection port and transfer line temperatures used were 250^{0}C and 280^{0}C respectively. The temperature programme initiated at 60^{0}C for 2 min hold, then it was raised at 15^{0}C per min to 160^{0}C, which was held for 0min, and then at 3^{0}C per min to 200^{0}C which was held for 1min, and again at 8^{0}C per min to 280^{0}C which was held for 6min. The mass spectrum of compounds present in sample was recorded with electron impact ionization energy 70eV over mass range 50-650Da amu.

RESULTS

The culture under present study, *Burkholderia* sp.VIMP04 (JQ867373) was protease and chitinase positive and cellulase negative. Based on HPLC,revealed organic acid profile of the strain VIMP04 is presented in a **Table 1** along with retention time (RT) and organic acid content in mg/100ml while peaks are indicated in **Figure 1**. In the culture filtrate, three organic acids were detected. The highest amount of organic acid produced by the *Burkholderia* sp.VIMP04 (JQ867373) was acetic acid which was followed by the formic acid and oxalic acid. The percent growth inhibition estimated via dual culture method by the strain VIMP04 against C. *paradoxa* was 20 percent. Both ethyl acetate and culture filtrate extracts under present investigation inhibited the growth of fungal pathogen C. *paradoxa* with zone of inhibitions (ZOI) 19mm (Standard deviation (SD) ± 2) and 25mm (SD ± 2), respectively.

The GC-MS analysis of ethyl acetate extract revealed 09 compounds. Gas chromatogram of ethyl acetate extract is presented in **Figure 2.** The compounds identified using NIST database are presented in **Table 2** along with retention time (RT), molecular formula (MF), and molecular weight (MW).Mass spectra of the compounds revealed are presented in **Figure 3 (3.1 to 3.9).**

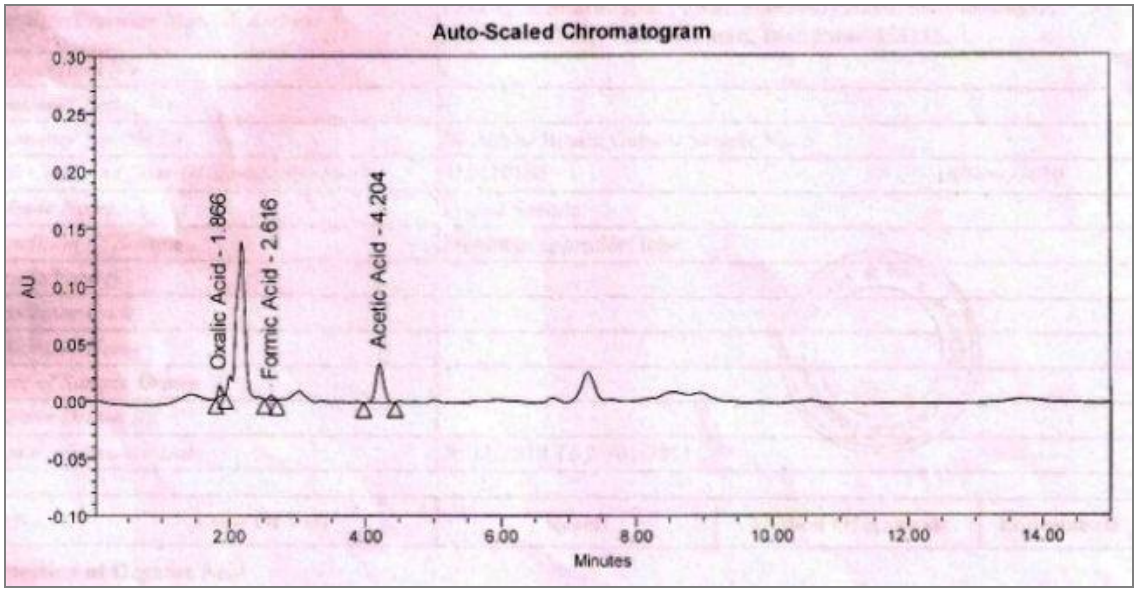

Figure 1: HPLC chromatogram.

Table 1: Organic acid profile of strain VIMP04 based on HPLC.

Sr. No.	Organic acids	RT (min)	Content (mg/100ml)
1	Oxalic acid	1.866	0.30
2	Formic acid	2.616	1.88
3	Pyruvic acid	-	–
4	Lactic acid	–	–
5	Citric acid	–	–
6	Gibberelic acid	–	–
7	Acetic acid	4.204	42.75

Note: "–" Not detected

Table 2: GC-MS profile of ethyl acetate extract.

Sr. No.	Name of compound	Mol.formula	MW	RT(min)
1	E-2-octadecadecen-1-ol	$C_{18}H_{36}O$	268	16.00
2	7,9-Di-ter-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	19.34
3	Phthalic acid butyl 2-pentyl ester	$C_{17}H_{24}O_4$	292	20.32
4	10-Heneicosene	$C_{21}H_{42}$	294	20.82
5	Eicosene 7-hexyl	$C_{19}H_{38}$	366	20.97
6	Cyclohexane (6-cyclopentyl-3-(3-cyclopentylpropyl)hexyl	$C_{25}H_{46}$	346	26.56
7	Tetratetracontane	$C_{44}H_{90}$	618	26.74
8	Cyclohexane 1,1-dodecylidenebis (4-methyl)	$C_{26}H_{50}$	362	33.18
9	Heptadecane 9-hexyl	$C_{23}H_{48}$	324	33.35

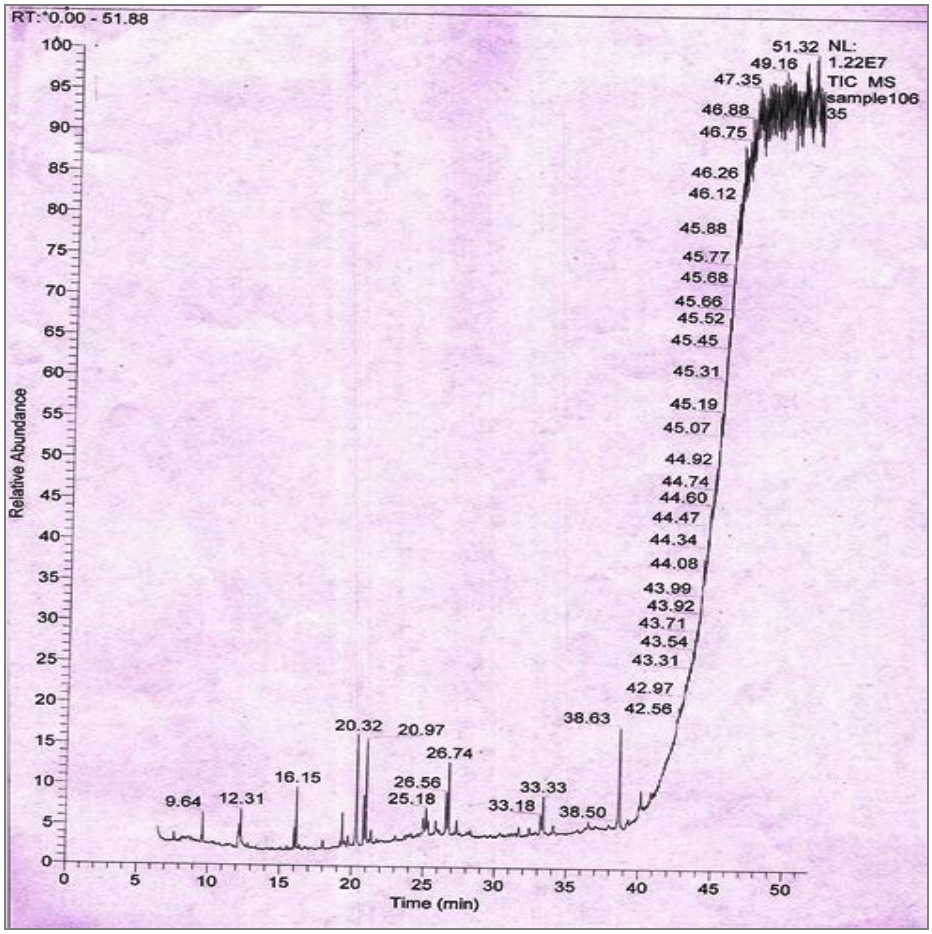

Figure 2: Gas chromatogram of ethyl acetate extract

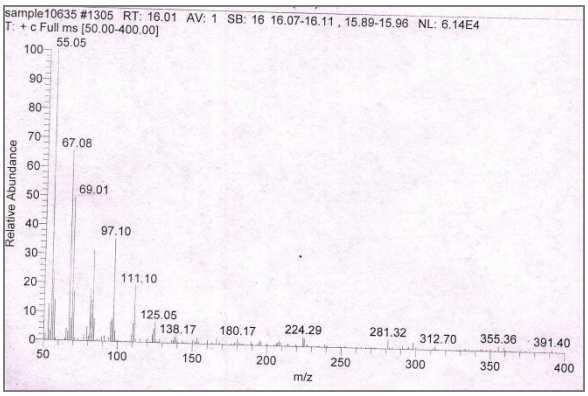

Figure 3: Mass spectra (MS) of the compounds.
Fig. 3.1: MS of E-2-octadecadecen-1-ol.

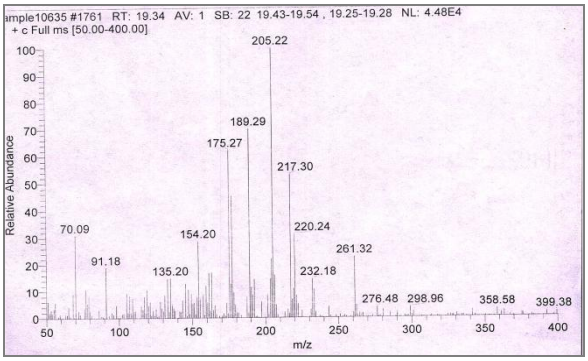

Fig. 3.2: MS of 7, 9-Di-ter-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-Dione.

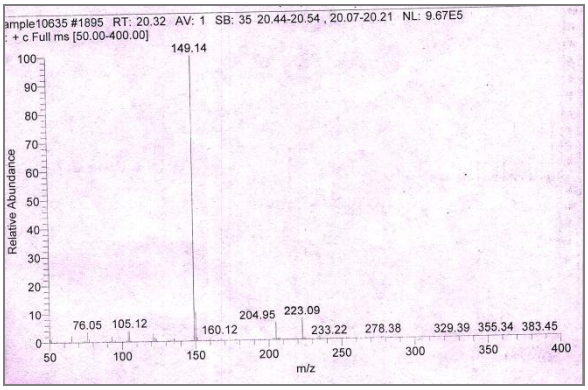

Fig. 3.3: MS of Phthalic acid butyl 2-pentyl ester

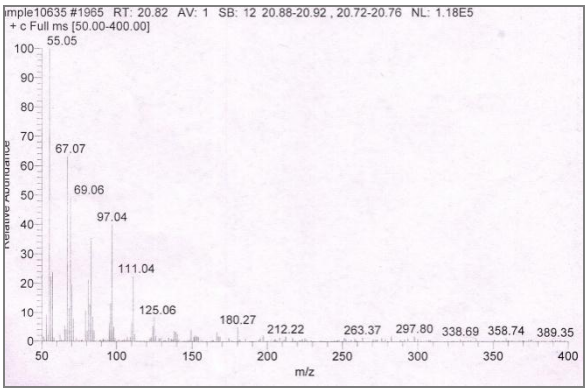

Fig. 3.4: MS of 10-Heneicosene.

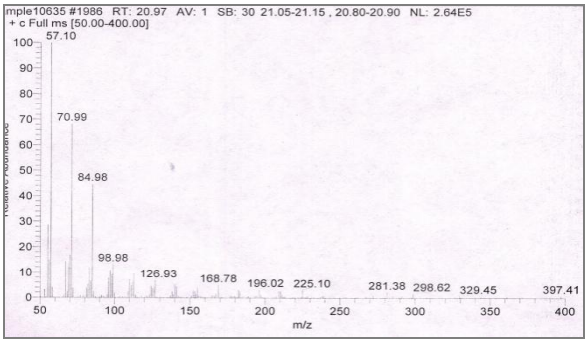

Fig. 3.5: MS of Eicosene 7-hexyl.

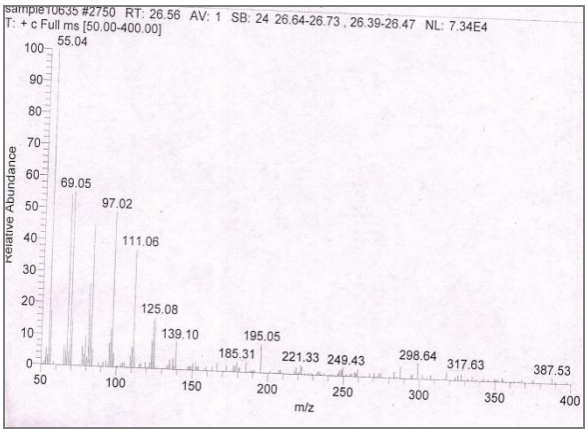

Fig. 3.6: MS of Cyclohexane (6-cyclopentyl-3-(3-cyclopentylpropyl)hexyl

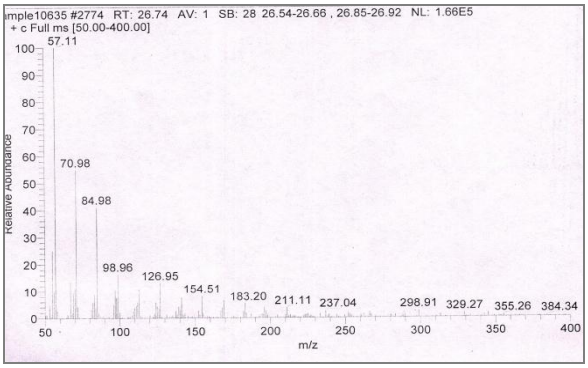

Fig. 3.7: MS of Tetratetracontane

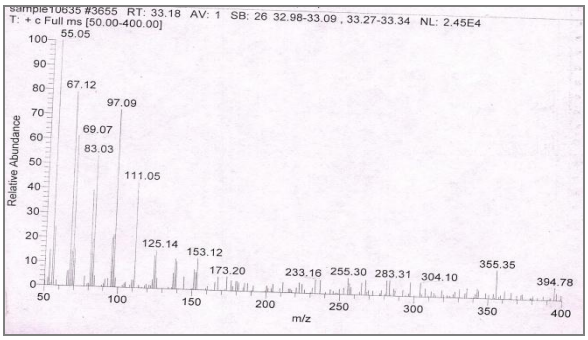

Fig. 3.8: MS of Cyclohexane 1, 1-dodecylidenebis (4-methyl).

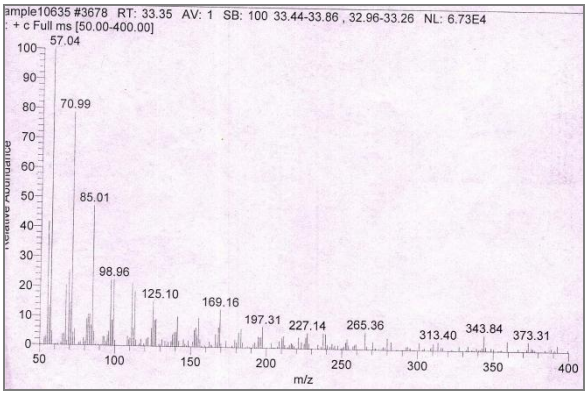

Fig. 3.9: MS of Heptadecane 9-hexyl.

DISCUSSION

ZOI recorded by the aqueous culture filtrate extract was found to be more. In the present study ZOIs recorded by the aqueous culture filtrate extract and ethyl acetate extract were not in harmony with the findings of Bhuvaneswari and Gobalakrishnan (2014) who reported more powerful antimicrobial activity oforganic solventsas compared to aqueous extracts. Fungal growth inhibition may be due to specific or non-specific metabolites, lytic enzymes, volatile compounds or other toxic substances (Sharma *et al.*2013). Lavermicocca *et al.* (2000) reported that lactic and acetic acid produced by bacterium played the most significant role in antifungal actions.The VIMP04 strain produced variety of organic acids as presented in Table 1and lytic enzymes such as chitinase and protease. These might be the reasons that the culture exhibited antifungal activities as supported by percent inhibition by dual culture method and ZOIs shown by both filtrate extracts. The ZOI exhibited by culture filtrate extract was more than the ZOI revealed by the ethyl acetate extract. This might be due to presence of more amounts of lytic enzymes, volatile compounds and other metabolites in concentrated and active states in culture filtrate rather than in the ethyl acetate extract. Similar outcomes were reported with other species of *Burkholderia* (Mahamuni[a], 2015; Mahamuni[b], 2015; Mahamuni *et al.*, 2017) against *Alternaria alternata* and *C. paradoxa*.

Many researchers reported the antimicrobial role of phenolic, alkaloid, carboxylic acid, hydrocarbon, ketone, ester, phthalate derivatives (Sultan *et al.* 2010).Presence of antimicrobial heptadecane was reported by Khairy and El-Kassas (2010) in ethyl acetate extract from blue green algae.Reported antifungal compounds produced by different species of *Burkholderia* were Phthalic acid butyl 2-pentyl ester, Tetratetracontane, 10-Heneicosene, Heptadecane 9-hexyl, E-2-octadecadecen-1-ol,

Cyclohexane 1,1-dodecylidenebis(4-methyl) and Cyclohexane (6-cyclopentyl-3-(3-cyclopentylpropyl) hexyl (Mahamuni[a], 2015; Mahamuni[b], 2015; Mahamuni *et al.*, 2017).Usha*et al.* (2015) documented antimicrobial compounds of marine *Streptomyces cacaoi* strain SU2 (JF730119) in the ethyl acetate extract by GC-MS such as phthalic acid butyl ester and 1 nonadecene derivatives. El-Baz*et al.* (2015) listed the presence of antimicrobial tetratetracontane in ethyl acetate extract of *Jatropacureas* leaves. Ahsan *et al.*(2017) reported Eicosane and dibutyl phthalate producing *Streptomyces*s train KX852460 having antifungal activity against *Rhizoctonia solani*. Results of the present investigation are in agreement with metabolites reported by above researchers. These components may affect fungal cell wall, proteins and nucleic acids. However variations at the level of antifungal activities, HPLC and GC-MS profiles can be explained on the basis of cultural differences, media composition, growth conditions, and diversity in antifungal metabolites.

CONCLUSIONS

The present study outcomes emphasized antifungal activity of *Burkholderia* sp.VIMP04 (JQ867373).Many of the bioactive principles recorded in the present study were not reported earlier. The GC-MS and HPLC profiles in combination may be unique for the culture under study. The culture may have potential to develop biopesticide.Field studies should be conducted in future to check the impact of *Burkholderia* sp.VIMP04 (JQ867373) in decreasing incidence of fungal soil borne diseases.

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