

ISOLATION OF POTENTIAL ANTIMICROBIAL METABOLITE FROM ENDOPHYTIC *BACILLUS AMYLOLIQUEFACIENS* DL06 OF CARNIVOROUS PLANT *DROSERA BURMANNII* VAHL.MADHUBANTI CHAUDHURI¹, PAUL AK¹, ARUNDHATI PAL^{2*}¹Microbiology Laboratory, Department of Botany, University of Calcutta, Kolkata, West Bengal, India. ²Post Graduate, Department of Botany, Serampore College, Hooghly, West Bengal, India. Email: arundhatipalcu@gmail.com

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ABSTRACT

Objectives: Exploitation of bacterial endophytes for production of antimicrobial substances has led to the discovery of novel natural metabolites of diverse chemical nature. The present study focuses attention toward optimization of cultural conditions for production of antimicrobial compound(s) by an endophytic bacterium DL06 followed by its extraction and partial purification.

Methods: The leaf endophytic bacterium *Bacillus amyloliquefaciens* DL06 (GenBank Accession no. MK696415, Microbial Culture Collection Accession no. 4186) isolated from carnivorous plant *Drosera burmannii* has been identified as a potent producer of antimicrobial metabolite following agar cup assay against several test bacterial and fungal strains. Cultural conditions for production of antimicrobials were optimized by "one variable at a time" method. The active fraction was isolated and purified partially using solvent extraction, thin-layer chromatography, and high performance liquid chromatography (HPLC) analysis.

Results: *B. amyloliquefaciens* DL06 produced maximum antimicrobial compound in tryptic soy broth and Davis-Mingoli's medium when grown under shake culture. Production of the antimicrobial metabolite has been optimized for the inoculum density, aeration, temperature, pH as well as carbon, and nitrogen sources. The antimicrobial metabolite was extracted from the cell-free culture filtrate in butanol and partially purified by silica gel column chromatography and HPLC.

Conclusions: The antimicrobial metabolite, tentatively identified as quercetin showed broad spectrum bioactivity affecting several fungi and a number of Gram-positive and Gram-negative bacteria.

Keywords: Carnivorous plant, *Drosera burmannii*, Bacterial endophytes, Antimicrobial metabolite, *Bacillus amyloliquefaciens*.

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INTRODUCTION

The search for new and effective antibiotics and chemotherapeutic agents has prompted over the last few decades due to the development of multidrug resistance in human pathogenic microorganisms. Moreover, due to a number of environmental issues, the use of synthetic chemicals demands their replacement by natural substances, particularly from microbial sources as an alternative to control human and plant pathogens [1].

The enormous diversity of microorganisms harnessed from wide range of ecological niches has long been utilized by the mankind for the production of huge number of pharmaceuticals including antimicrobial substances [2]. Endophytic microorganisms residing inside the plants without producing any visible damage to the host plant are relatively under explored microbial population [3]. These hidden microbial communities have received substantial attention in recent years for their ability to produce novel bioactive secondary metabolites which are of special importance not only for the benefit of host plant but also for human beings as successful source of drugs. Functional metabolites produced by the endophytic microbes have a great potential for application in agricultural, food, pharmaceutical, and biotechnological industries [1,4]. Although many of these endophytic natural products have been demonstrated to exert antioxidant, antidiabetic, and immunosuppressive effects, a significant portion of them was established as antimicrobials [1,5-8].

Carnivorous plants show unique trapping organs for insect prey digestion and nutrient uptake. They have attracted the attention of the scientific community in the recent past because of distinctive plant

metabolites and novel products. Species of *Nepenthes* and *Drosera* have been reported to be used traditionally in India and Southeast Asian countries for their therapeutic activity in treatment of gastrointestinal and lung ailments [9,10]. Moreover, Buch *et al.* [11] have demonstrated antibacterial activity in the digestive fluid of *Nepenthes* spp. as well as from the fungal endophytes isolated from the fluid. Likewise, the endophytic fungi from *Nepenthes ampullaria* and *Nepenthes mirabilis* showed inhibition against a number of bacterial and fungal species [12,13]. However, the potentials of endophytic bacteria of carnivorous plants for production of antimicrobial metabolites have not been explored adequately and deserve due attention.

We have made preliminary investigations on the bioactive potentials of bacterial endophytes of *Drosera burmannii* [14,15]. In the present study, an attempt has been made to evaluate the production of antimicrobial metabolite by *Bacillus amyloliquefaciens*, a leaf endophyte of *D. burmannii*, and assess the *in vitro* efficacy of the metabolite against selected bacterial and fungal test strains.

METHODS**Source and maintenance of bacterial cultures**

The bacterium *Bacillus* DL06, a leaf endophyte of carnivorous plant *D. burmannii* Vahl., was isolated previously [15] and used throughout this study. The bacterium was maintained on slopes of nutrient agar by repeated sub-culturing.

The test organisms used for assessment of antimicrobial activity include *Acinetobacter baumannii* MTCC 1425, *Bacillus cereus* MTCC 1272, *Bacillus subtilis* MTCC 441, *Escherichia coli* MTCC 1687, *Klebsiella*

pneumoniae MTCC 530, *Proteus vulgaris* MTCC 426, *Pseudomonas aeruginosa* MTCC 1638, *Pseudomonas cepacia* MTCC 4684, *Salmonella typhimurium* MTCC 3224, *Staphylococcus aureus* MTCC 2943, *Staphylococcus epidermidis* MTCC 3383, *Staphylococcus haemolyticus* MTCC 435, *Vibrio cholerae* O139; *Alternaria solani* MTCC 2101, *Aspergillus niger* MTCC 281, *Curvularia lunata* MTCC 2030, *Fusarium oxysporum* MTCC 1755, *Penicillium citrinum* MTCC 1256, *Rhizoctonia solani* MTCC 4633, *Saccharomyces cerevisiae* MTCC 170, *Saccharomyces boulardii* CNCM I-745, *Sclerotium rolfsii*, and *Trichoderma viride*. While the bacterial and fungal cultures were maintained on nutrient agar and Czapek Dox agar, respectively, yeast extract peptone dextrose agar was used for *Saccharomyces* only.

Characterization and identification of the endophytic bacterial isolate

The bacterium DL06 was characterized morphologically and physio-biochemically according to standard microbiological methods [16]. Exponentially growing bacterial cells in nutrient broth was used for scanning electron microscopy (Carl Zeiss Zeiss Evo 18). Antibiotic sensitivity pattern was determined following the Kirby-Bauer disc diffusion method [17]. The diameter of inhibition zones was measured to the nearest mm and the organism was categorized as resistant, intermediate, and sensitive following DIFCO Manual 10th edition [18].

The 16S rRNA gene sequence analysis was carried out using two universal primer sets 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTACGACTT-3'). Consensus sequence of 16S rDNA was generated and compared with closely related neighbor sequences retrieved from the NCBI database using BLAST search. Phylogenetic analysis was performed using the software package MEGA 6.0 after obtaining multiple alignments of the data available from the public databases by Thompson et al. [19].

Time-course of growth and production of antimicrobials

Erlenmeyer flasks (250 ml) containing 50 ml of mineral salts medium were inoculated with overnight grown culture and incubated at 32°C under continuous shaking (120 rpm) for 144 h. Samples were withdrawn at regular interval for determination of growth and antimicrobial activity. Growth was measured by estimating the cell dry weight and expressed as g/L. Antimicrobial activity of the cell-free culture filtrate was assessed by agar cup assay following passage through membrane filter (0.2 µm) and freshly grown cultures of *E. coli* and *A. niger* were used as test organisms.

Determination of optimum conditions for production of antimicrobial metabolites

To determine the optimum conditions for production of antimicrobial metabolite, one variable at a time method was used and variations were made in the composition of growth media, inoculum density, aeration, temperature, pH, carbon, and nitrogen sources. Growth and antimicrobial activity were assessed following the method described above.

Isolation and partial purification of the antimicrobials

The bacterium was grown in mineral salts medium under optimized cultural conditions. The antimicrobial metabolite was obtained from the cell-free culture filtrate following solvent extraction procedure using petroleum ether, benzene, diethyl ether, dichloromethane, ethyl acetate, dimethyl sulfoxide, chloroform, and butanol. Individual solvent fractions were evaporated to dryness under reduced pressure, dissolved in sterile distilled water, and assayed for antimicrobial activity by agar cup assay. Preparative thin-layer chromatography (TLC) of the active solvent fraction was performed using a number of solvent systems, spots were detected under ultraviolet light, and bioactivity was assayed as described above.

Butanol extraction was carried out from 1 L of cell-free culture filtrate and concentrated to 30 ml. For column chromatography, the concentrated butanol fraction (30 ml) was loaded in a silica gel

column (mesh size 60–120, 20 mm×350 mm) pre-equilibrated with chloroform:methanol (1:3) mixture. The metabolites were eluted with chloroform:methanol (1:3) as the solvent system at a flow rate of 1 ml/min and fractions of 5 ml each were collected. Individual fractions were assayed for antimicrobial activity following evaporation and dissolution in sterile distilled water.

The pooled active fractions obtained from the silica gel column were evaporated to dryness, dissolved in 2 ml sterile distilled water, and subjected to high performance liquid chromatography (HPLC) using Agilent1290 Infinity II with pump model G1328 C and detector model G 7115 A. The C18 column (4.6 mm×250 mm) was used and peaks were detected at 280 nm. Phosphoric acid (0.5%) and methanol (90%) were used as mobile phases for gradient run of 15 min at a flow rate of 2 ml/min and injection volume of 100 µl. The fractions were collected with Agilent Infinity II Open LAB Chemstation Workstation model M830IAA and checked for antimicrobial activity following the usual agar cup assay.

RESULTS

Characterization and identification of isolate DL06

The endophytic isolate DL06 formed rough, white colonies on nutrient agar. Bacterium DL06 is Gram-positive, aerobic, motile rod (Fig. 1), forming endospores, and produced intra- and extracellular enzymes such as catalase, oxidase, amylase, nitrate reductase, pectinase, and protease. The optimum temperature and pH for growth were 32°C and 7, respectively. It tolerates a maximum concentration of 3% NaCl. The isolate could utilize as well as ferment a number of carbon sources. It showed resistance to polymyxin B, vancomycin, and chloramphenicol but was sensitive to erythromycin, ampicillin, ciprofloxacin, rifampicin, gentamycin, trimethoprim, tetracycline, chlortetracycline, novobiocin, and norfloxacin (Table 1). These morphological and physio-biochemical features of the isolate DL06 were compared with the standard descriptions available in the Bergey's Manual of Determinative Bacteriology [20] and was assigned to the genus *Bacillus*.

The nucleotide sequence of the 16S rRNA gene (1456 bp) of the bacterium DL06 was found to have 99% similarity with *B. amyloliquefaciens* and designated as *B. amyloliquefaciens* DL06. The nucleotide sequence of 16S rRNA gene of the strain DL06 has been submitted to the GenBank database of NCBI with an accession number of MK 696415 and the live culture has been deposited to the Microbial Culture Collection (MCC) and National Centre for Microbial Resource, Pune, India, under the accession number MCC 4186 (Fig. 2).

Time-course of growth and production of antimicrobials

The growth-associated production of antimicrobial metabolite from *B. amyloliquefaciens* DL06 was evaluated by growing bacterium in

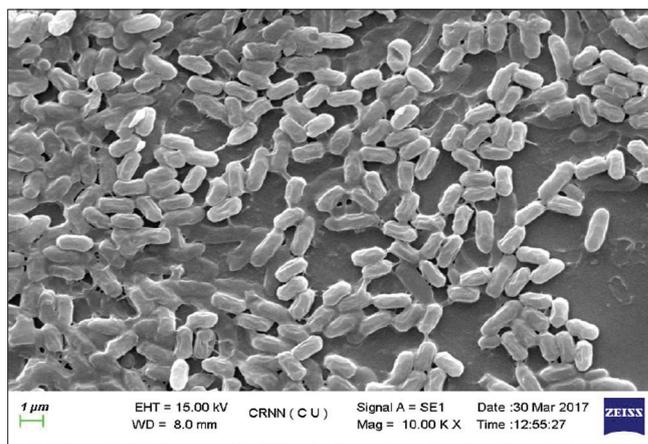


Fig. 1: Scanning electron micrograph showing morphology of cells of bacterial isolate DL06

Table 1: Morphological and physio-biochemical characteristics of the endophytic bacterial isolate DL06

Character	Response	Character	Response	Response
Colony	Rough, white	Fermentation and utilization of	Fermentation	Utilization
Cell	Rods, 2.5–3 µm×1.2–1.6 µm	Glucose	+	+
Gram nature	Gram +ve	Maltose	-	+
Motility	+	Sucrose	-	+
Endospore formation	+, central	Mannitol	+	+
Production of catalase	+	Sorbitol	+	+
Caseinase	+	Fructose	+	+
Oxidase	+	Galactose	+	+
Amylase	+	Lactose	+	-
Gelatinase	+	Rhamnose	-	-
Pectinase	+	Raffinose	-	+
Urease	-	Arabinose	+	+
Nitrate reductase	+	Aldonitol	-	-
Cellulase	-	Dulcitol	-	-
Lipase	-	Trehalose	-	+
Indole production	-	Inositol	-	-
Citrate utilization	-	Mannose	-	+
Optimum growth temp.	32°C			
Optimum growth pH	7	Antibiotic susceptibility	Pb ^r , C ^r , E ^s , Amp ^s , Cip ^s , Rif ^r , Gen ^s , Tri ^s , Van ^r , Tet ^s , Ct ^s , Nov ^s , Nor ^s	
Maximum NaCl% tolerance	3%			

Antibiotics: E: Erythromycin, Pen: Penicillin G, C: Chloramphenicol, Amp: Ampicillin, Cip: Ciprofloxacin, Rif: Rifampicin, Gen: Gentamycin, Tri: Trimethoprim, Van: Vancomycin, Tet: Tetracycline, Ct: Chlortetracycline, Nov: Novobiocin, Nor: Norfloxacin, s: Sensitive, r: Resistant, + indicates positive response, - indicates negative response

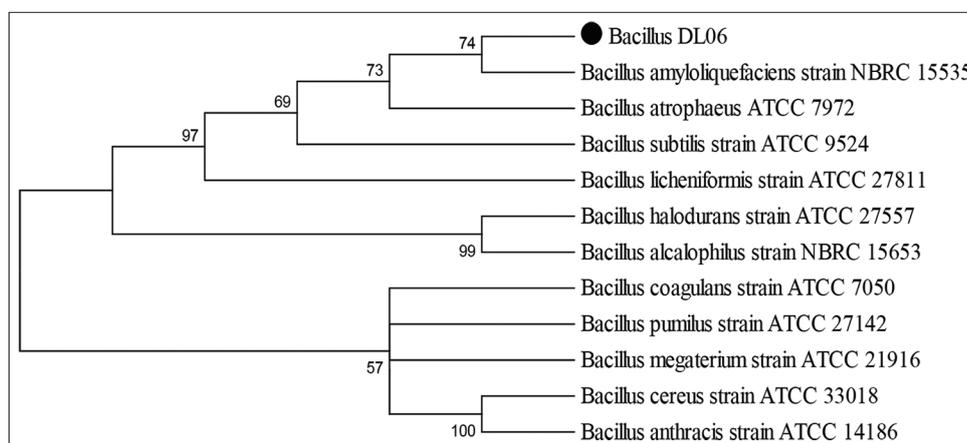


Fig. 2: Phylogenetic relationship of the endophytic bacterial isolate DL06 with other closely related species based on 16S rRNA gene sequence analysis

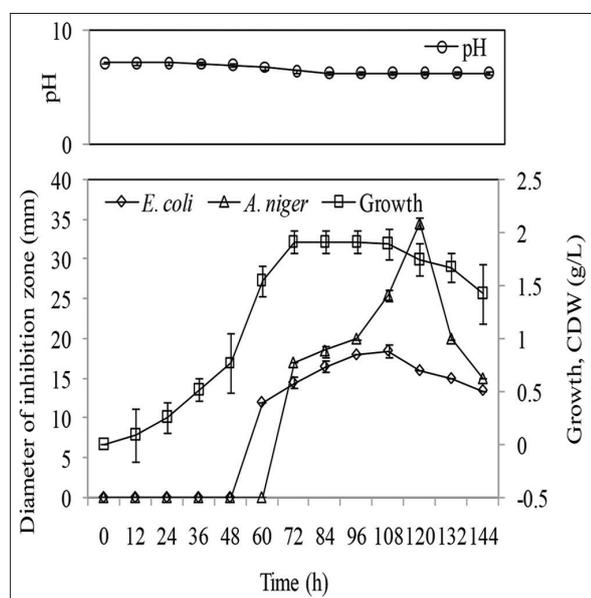


Fig. 3: Time course of growth and production of antimicrobial metabolite by *Bacillus amyloliquefaciens* DL06

mineral salts medium under shake-flask culture and bioactivity was assessed following agar cup assay against *E. coli* and *A. niger*. Production of antimicrobial metabolite was found to initiate during the active phase of growth which attained its highest titer (diameter of inhibition zone 33 mm against *A. niger*) during the late stationary phase (120 h) (Fig. 3).

Spectrum of bioactivity of *B. amyloliquefaciens* DL06

The cell-free culture filtrate from *B. amyloliquefaciens* DL06 was evaluated for antimicrobial spectrum determination following agar cup assay using test bacterial and fungal strains (Table 2). The metabolite showed broad spectrum of activity affecting *B. cereus*, *S. haemolyticus*, *S. epidermidis*, *P. aeruginosa*, and *K. pneumoniae* among the bacteria and *A. solani*, *C. lunata*, *S. rolfssii*, *R. solani*, *F. oxysporum*, *T. viride*, and *S. bouldardii* among the fungi.

Optimization of cultural conditions

During the present study, a number of cultural conditions affecting growth and production of antimicrobial metabolites were optimized using one variable at a time. The variables include culture media of different composition, inoculum density, aeration, temperature, pH, carbon, and nitrogen sources. Among different complex and synthetic media tested, bioactivity against *A. niger* was found to be high in tryptic soy broth and Davis-Mingioli's medium followed by mineral salts medium (MSM), Straw infusion medium (SI), and Gause's synthetic medium (GSM) (Fig. 4).

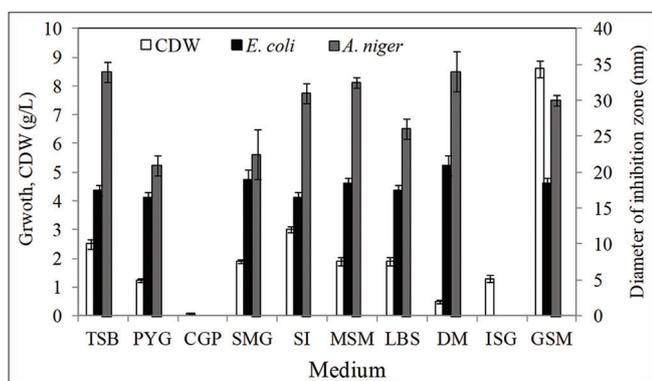


Fig. 4: Effect of different culture media on growth and production of antimicrobial metabolite by *Bacillus amyloliquefaciens* DL06. TSB: Tryptic soy broth, PYG: Peptone yeast extract glucose broth, CGP: Casamino acid glucose peptone broth, SMG: Soybean meal glucose medium, SI: Straw infusion medium, MSM: Mineral salts medium, LBS: Lindenbein synthetic medium, DM: Davis-Mingoli's medium, ISG: Inorganic salt glucose medium, GSM: Gause's synthetic medium

Table 2: Antimicrobial spectrum of the cell-free culture filtrate of *Bacillus amyloliquefaciens* DL06

Test organism	Diameter of inhibition zone (mm)
Bacteria	
<i>Acinetobacter baumannii</i> MTCC 1425	NI
<i>Bacillus cereus</i> MTCC 1272	14.5±0.71
<i>Bacillus subtilis</i> MTCC 441	14.0±1.41
<i>Burkholderia cepacia</i> MTCC 4684	NI
<i>Escherichia coli</i> MTCC 1687	17.5±0.71
<i>Klebsiella pneumoniae</i> MTCC 530	12.5±0.71
<i>Proteus vulgaris</i> MTCC 426	NI
<i>Pseudomonas aeruginosa</i> MTCC 1688	14.5±0.71
<i>Salmonella typhimurium</i> MTCC 3224	NI
<i>Staphylococcus aureus</i> MTCC 2943	13.0±2.82
<i>Staphylococcus epidermidis</i> MTCC 3383	14.5±0.52
<i>Staphylococcus haemolyticus</i> MTCC 435	13.5±0.55
<i>Vibrio cholerae</i> O139	NI
Fungi	
<i>Alternaria solani</i> MTCC 2101	31.5±0.55
<i>Aspergillus niger</i> MTCC 281	32.5±0.71
<i>Curvularia lunata</i> MTCC 2030	32.5±0.71
<i>Fusarium oxysporum</i> MTCC 1755	15.5±0.71
<i>Penicillium citrinum</i> MTCC 1256	NI
<i>Rhizoctonia solani</i> MTCC 4633	24.5±0.71
<i>Saccharomyces boulardii</i> CNCM I-745	10.5±0.52
<i>Saccharomyces cerevisiae</i> MTCC 170	24.0±1.41
<i>Sclerotium rolfsii</i>	20.0±0.71
<i>Trichoderma viride</i>	22.5±0.52

^aAntimicrobial activity was determined following standard agar cup assay method. NI: No inhibition, Values represent mean of triplicates±standard deviation

Table 3: Optimization of cultural conditions for growth and production of antimicrobial metabolite by *Bacillus amyloliquefaciens* DL06

Parameter	Test range	Optimum condition	Growth, CDW (g/L)	Diameter of inhibition zone (mm)	
				<i>Escherichia coli</i>	<i>Aspergillus niger</i>
Inoculum dose (% v/v)	0.5-2	1.5	0.58±0.11	21.5±0.71	34.5±0.71
Aeration (CVF)	1:10, 1:5, 2:5, 3:5, 4:5	1:5	0.7±0.08	21.5±0.71	34±1.41
Temperature (°C)	28-42	32	0.75±0.11	20.5±0.71	34.5±0.71
pH	5-10.6	6.6	0.81±0.08	23.5±0.71	34.5±0.71
Carbon source	Glu, fru, gal, lac, mal, suc, mann, raff, sor, ara, ino, man	Glu	0.5±0.11	20.5±0.71	34.5±0.71
Glucose (% w/v)	1.6-2.4	2.2	0.56±0.02	22.5±0.71	35±1.41
Nitrogen source	Pep, Tryp, Cas, YE, NH ₄ Cl, (NH ₄) ₂ SO ₄ , NaNO ₃ , NH ₄ NO ₃	Tryp	0.69±0.11	21±1.41	35±1.41
Tryptone (g/L, w/v)	0.6-1.4	1.2	1.02±0.03	22.5±0.71	36.5±0.71

Values represent mean of triplicate experiments±standard deviation; CVF: Culture volume by flask volume ratio; glu – glucose, gal – galactose, suc – sucrose, ara – arabinose, fru – fructose, tre – trehalose, sor – sorbitol, man – mannose, mann – mannitol; Pep – peptone, Cas – casamino acid, Tryp – tryptone, YE – Yeast extract

Maximum growth and production of antimicrobial metabolite occurred at an inoculum dose of 1.5% (v/v) and at culture volume:flask volume ratio (CVF) of 1:5. The optimum temperature and pH for antimicrobial activity were recorded at 32°C and 6.6, respectively. Among the 12 different carbon sources tested, glucose at a concentration of 2.2% (w/v) was found to be most effective for production of antimicrobial metabolite. Likewise, tryptone was the best utilized nitrogen source for bioactivity and maximum growth as well as antimicrobial activity was observed at a concentration of 1.2 g/L (Table 3).

Isolation and partial purification of the antimicrobials

The antimicrobial metabolites produced by *B. amyloliquefaciens* DL06 in mineral salts medium was extracted from the cell-free culture filtrate using a number of solvents. The antimicrobial metabolite was best extracted in butanol; however, dichloromethane and ethyl acetate as extractants were not that efficient (Table 4).

The butanolic extract when subjected to TLC using a number of solvent systems, maximum number of spots was detected in the chloroform:methanol (1:3) mixture. Preparative TLC revealed that the third spot (C₃) with R_f value of 0.84 was having bioactivity. The second spot (C₂) with more or less identical R_f value (0.87) as detected in ethanol:water:chloroform (4:4:2) mixture also showed a comparatively lesser degree of inhibition (Table 5).

Further, the concentrated butanolic extract of the cell-free culture filtrate was subjected to column chromatography (silica gel, 60-120 mesh size) using chloroform:methanol (1:3) mixture as the solvent system. Fractions, 5 ml each were collected, evaporated to dryness and assayed for antimicrobial activity after dissolving in sterile water.

Bioactivity obtained in fractions 7 and 8 were pooled and subjected to HPLC (Agilent1290 Infinity II) analysis. Six distinct peaks with retention time were visualized in the chromatogram (Fig. 5). The compound with retention time 12.5 min showing antimicrobial activity against *E. coli* and *A. niger* was tentatively identified as quercetin.

DISCUSSION

The increasing demand for discovery of new antimicrobials has led the scientific community to explore the vast diversity of endophytic microorganisms in plants as potential bioresource for bioactive metabolites [1]. Several studies have reported the isolation of large number of chemically diverse group of antimicrobials from endophytes of different plant sources [13,21-23].

Association of microbial communities in the internal environment of carnivorous or insectivorous plants has received attention only in the last few decades. Both culture dependent and culture independent studies have established the occurrence of endophytic bacteria and fungi in these spectacular plants [24-28]. While preliminary reports

on antimicrobial activities of fungal endophytes of *N. mirabilis* and *N. ampullaris* [12] and the bacterial endophytes of *Drosera* and *Utricularia* [14,15] have recently been published, no in depth studies on their metabolites have been undertaken so far. The present study is based on the production, partial characterization, and optimization of antimicrobial metabolite from a leaf endophytic bacterium *Bacillus* DL06 from *D. burmannii*. The Gram-positive, endospore forming motile rod-shaped bacterium was identified as *Bacillus amyloliquefaciens* DL06 (GenBank Accession number MK696415; MCC Accession no. 4186) based on phenotypic features and 16S rRNA gene sequence

Table 4: Solvent extraction of antimicrobial substance produced by *Bacillus amyloliquefaciens* DL06^a

Solvent	Diameter of inhibition zone (mm)	
	Test organism	
	<i>Escherichia coli</i>	<i>Aspergillus niger</i>
Petroleum ether	NI	NI
Benzene	NI	NI
Diethyl ether	NI	NI
Dichloromethane	12.5±0.71	14.5±0.71
Ethyl acetate	14.5±2.82	15.5±0.71
Dimethyl sulfoxide	NI	NI
Chloroform	NI	NI
Butanol	21.5±0.71	35.5±2.81

^aAntimicrobial metabolite was extracted from the culture filtrate of the isolate after 120 h of growth and assayed against test organisms following agar cup method. NI: No inhibition, Values represent mean of triplicates±standard deviation

analysis (Fig. 1 and 2 and Table 1). Occurrence of *B. amyloliquefaciens* as endophyte is not new and has already been reported from different plant sources (Table 6).

Cell-free culture filtrate of *Bacillus* DL06 was found to inhibit the growth of *B. cereus*, *S. haemolyticus*, *S. epidermidis*, *P. aeruginosa*, and *K. pneumoniae* among the bacteria tested and *A. solani* and *C. lunatas* among the fungi (Table 2). Comparison of antimicrobial activities of the present isolate DL06 with those of endophytic *B. amyloliquefaciens* reported previously [29-34] was carried out (Table 6). While the metabolites produced by *B. amyloliquefaciens* BZ6-1, Blu-v-2, August M-1, M-2, and Halycon1 showed antifungal activities against variety of phytopathogens [29,30], *B. amyloliquefaciens* PEBA-20 endophytic to poplar exhibited both antifungal and antibacterial activities [31]. Leaf endophyte *B. amyloliquefaciens* HY-10 from *Hyptis suaveolens* demonstrated activity against clinical pathogens including *Shigella dysenteriae* and *Candida* spp. [32]. Bhoonobong et al. [34] reported isolation and purification of antibacterial metabolites from *B. amyloliquefaciens* UD25 that inhibited several bacterial test strains including *S. aureus* (MRSA). A novel antimicrobial protein was reported from endophytic *B. amyloliquefaciens* that showed biocontrol of *Fusarium chlamydosporum* [35]. Recently, Wang and Wang [33] showed that resistance of sweet potato was elicited against two fungal pathogens due to *in vitro* occurrence of endophytic *B. amyloliquefaciens* YTB1407.

Time-course studies for growth and production of antimicrobial metabolite were carried out (Fig. 3). Suitable media for production of the metabolite by *B. amyloliquefaciens* DL06 (Fig. 4) and the optimum cultural conditions were determined (Table 3). The bioactive compound

Table 5: Preparative thin-layer chromatography of the butanol fraction of cell-free culture filtrate of *Bacillus amyloliquefaciens* DL06^a

Solvent system	No. of spots	R _f value	Diameter of inhibition zone (mm)		
			Test organism		
			<i>Escherichia coli</i>	<i>Aspergillus niger</i>	
Ethanol:water:chloroform (4:4:2)	C ₁	0.27	NI	NI	
	C ₂	0.87	18.5±0.71	30.5±0.71	
	Chloroform:methanol (1:3)	C ₁	0.69	NI	NI
		C ₂	0.77	NI	NI
Benzene:ethyl acetate (1:9)	C ₃	0.84	23.5±0.71	36.5±0.71	
	C ₄	0.91	NI	NI	
	C ₁	0.25	NI	NI	
		0.34	NI	NI	

^aIndividual spots were eluted and antimicrobial activity assayed against test organisms following agar cup method. NI: No inhibition, Values represent mean of triplicates±standard deviation

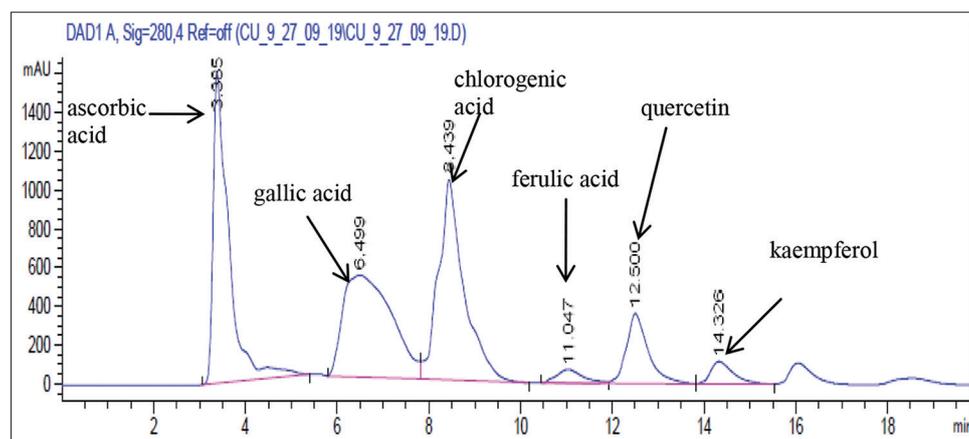


Fig. 5: High performance liquid chromatography chromatogram of the antimicrobial metabolites produced by *Bacillus amyloliquefaciens* DL06

Table 6: Comparison of *Bacillus amyloliquefaciens* DL06 with other endophytic strains with respect to their source, antimicrobial metabolites, and activities

<i>Bacillus amyloliquefaciens</i> strains	Source of endophyte	Isolation medium	Antimicrobial spectrum		Test method used	Metabolite identified/ chemical nature	Reference
			Bacteria	Fungus			
PEBA 20	Stem of <i>Populus tomentosa</i>	Nutrient agar	<i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Bacillus megaterium</i>	<i>Saccharomyces cerevisiae</i> , <i>Xanthomonas campestris</i> , <i>Ralstonia solanacearum</i> , <i>Botryosphaeria dothidea</i>	Disc diffusion and agar-well diffusion assay	NR	[31]
BZ6-1	Peanut stem	Luria-Bertani agar	NR	<i>Ralstonia solanacearum</i>	Agar-well diffusion assay	Homologue of surfactin and fengycin A	[30]
UD25	Medicinal plant <i>Memecylon edule</i>	Nutrient agar	<i>Staphylococcus aureus</i> (MRSA), <i>Pseudomonas aeruginosa</i> , <i>Bacillus cereus</i> , <i>Escherichia coli</i>	NR	Agar-well diffusion assay	Presence of carboxylic acids, esters, anthraquinone, alkaloids, carbonyl, thiol, organohalogen	[34]
Blu-v2, August-M1, August-M2, Halycon1	Seed of ornamental plants	Trypticase soy agar	NR	<i>Alternaria alternata</i> , <i>Fusarium oxysporum</i> , <i>Colletotrichum crassipes</i> <i>Candida</i> spp.	Cross-streak method	Isoforms of surfactins, iturins, and fengycins	[29]
HY-10	<i>Hyptis suaveolens</i>	Nutrient agar	<i>Escherichia coli</i> , <i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , <i>Shigella dysenteriae</i>		Disc diffusion assay	Absorbance at 254 nm ($\lambda_{max}=1.541$)	[32]
YTB1407	<i>Ipomoea batatas</i>	NR	NR	<i>Fusarium solani</i> , <i>Ceratocystis fimbriata</i>	<i>In vitro</i> and pot trial assays	Expression of SA-responsive NPR 1 and PR 1 genes in the host	[33]
DL06	Leaf of <i>Drosera burmannii</i>	Nutrient agar	<i>B. subtilis</i> , <i>E. coli</i> , <i>Staphylococcus aureus</i> , <i>Staphylococcus haemolyticus</i> , <i>Staphylococcus epidermidis</i> , <i>Pseudomonas aeruginosa</i> , <i>Klebsiella pneumoniae</i>	<i>Aspergillus niger</i> , <i>Saccharomyces cerevisiae</i> , <i>Alternaria solani</i> , <i>Sclerotium rolfsii</i> , <i>Rhizoctonia solani</i> , <i>Curvularia lunata</i> , <i>Fusarium oxysporum</i>	Agar cup diffusion assay	Tentatively quercetin	Present study

NR: Not reported

was extracted in butanol (Table 4), preparative TLC was performed (Table 5) followed by HPLC analysis (Fig. 5), and the active fraction was determined tentatively as quercetin. The plant flavonol quercetin belongs to the flavonoid group of polyphenols. Inhibitory action of quercetin has been observed against *S. aureus*, *P. aeruginosa*, *P. vulgaris*, and *E. coli* [36,37]. Quercetin also showed antifungal effects against *Cryptococcus* spp. and *Candida* spp. [38-40]. Several reports document the production of different flavonoids from microbial endophytes. Quercetin was detected in secondary metabolites of endophytic bacteria of *Cosmos caudatus* leaf which showed anticancer and antimicrobial properties [41]. Endophytic fungi from *Pinus roxburghii* produce flavonoids having antimicrobial properties [42]. Flavonoids having bioactive potentials were identified from endophytic fungi of the medicinal plant *Tragia involucrata* which included vanillin, quercetin, caffeic acid, and ferulic acid [43]. As of now, Lee et al. [12] have shown

antimicrobial properties of the fungi endophytic to *Nepenthes* spp. It had inhibitory activities against *Streptococcus pyogenes*, *Enterococcus faecalis*, and *Ganoderma boninense*.

CONCLUSIONS

The present study reports on the production of antifungal and antibacterial compound by a leaf endophyte *B. amyloliquefaciens* isolated from the carnivorous plant *D. burmannii* Vahl. Production of antimicrobial compound by the endophytic bacterial isolate was further optimized. The antimicrobial compound was purified and chemical nature of the active fraction was tentatively determined as quercetin. The research deserves special attention for application of bacterial endophytes from carnivorous plants in the field of pharmaceutical biotechnology.

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Author MC performed the experiments and prepared the draft manuscript. Author AKP designed the experiments. Author AP managed the analyses of result and literature survey. AKP and AP prepared the final manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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