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Antibacterial Activity of Endophytic *Bacillus safensis* Isolated from *Ophioglossum reticulatum* L.

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AM and RD performed the experiments, tabulated the results and prepared the draft manuscript. Author AP managed literature searches and analysis of experimental data. Author AKP designed the experimental protocol and prepared the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study is aimed to explore the potential of endophytic bacterial population of *Ophioglossum reticulatum* L., a pharmacologically important perennial fern, for production of antimicrobial compounds.
Place and Duration of Study: The study was conducted in the Microbiology Laboratory, Department of Botany, University of Calcutta, Kolkata, India, between March 2016 and August 2016.
Methodology: Phenotypically distinct bacterial endophytes were isolated from surface sterilized segments of *O. reticulatum* L. and screened for the production of antimicrobial metabolites following cross-streak and agar-cup assay methods. The most potent antibacterial isolate was characterized in terms of morphological, physio-biochemical features, and 16S rDNA sequence analysis. The antibacterial antibiotic produced in tryptic soy broth was isolated from the fermented medium in ethyl acetate and partially purified by preparative TLC. The nature and antimicrobial spectrum of the antibiotic was determined following standard biochemical and microbiological methods.
Results: The potent endophytic bacterial isolate OPL 19 was identified as *Bacillus safensis* (GenBank accession number KY029081) following a polyphasic approach. The antibacterial

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compound produced by *Bacillus* OPL 19 was found to be relatively thermostable, non-polar, and lipoidal in nature showing distinct absorption peaks at 220 and 235 nm. In addition it showed broad spectrum of activity inhibiting wide variety of Gram-positive and Gram-negative bacteria including *Acinetobacter baumannii, Bacillus subtilis, Cellulosimicrobium cellulans, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* etc.

Conclusion: This study not only indicated the endomicrobiota as a potential resource for novel antimicrobials but also highlighted the fact that the therapeutic properties of *O. reticulatum* L. could be correlated with its endophytic association.

Keywords: Ophioglossum reticulatum L.; antimicrobial spectrum; Bacillus safensis; extraction of antibiotic; antibacterial activity.

1. INTRODUCTION

Ophioglossum, commonly known as adder's-tongue ferns with about 25-30 temperate and tropical species belongs to the family Ophioglossaceae of the order Ophioglossales. The sporophytic plant is well differentiated into a sub-terranean rhizome with roots and a single leaf bearing a simple, stalked, cylindrical sporangiferous spike with two rows of embedded sporangia. They are usually terrestrial with few exceptional epiphytic species and are widespread in distribution. In India, *Ophioglossum reticulatum* L. is mainly found in Tamil Nadu, Kerala and hills of West Bengal.

The pteridophytes are known to human being for their therapeutic and medicinal values. Caius [1] is probably the first to describe the medicinal uses of some ferns of India. Besides this, the medicinal values of ferns in India have been reviewed and highlighted by several others [2,3]. *Ophioglossum* spp. in general are no exception to this generalization and *O. reticulatum* L.*, O. costatum* R. Br.*, O. gramimeum* Willd. and *O. nudicaule* L. have been used in menstrual disorders and on burns as cooling agent. *O. gramimeum* has also been reported to possess antimicrobial, anticancer, antiseptic, detergent and vulnerary properties [4,5,6].

Endophytic microorganisms are found to inhabit symbiotically in the living tissues of almost every plant on earth without producing any harmful or deleterious effect on the host plant [7]. These endophytes may produce variety of metabolites for potential use in medicine, industry and agriculture and include novel antibiotics, antimicrobials, immunosuppressants, anticancer compounds etc. Studies have revealed that the antimicrobial compounds isolated from endophytes can be used as drugs and also as food preservatives. Castillo et al. [8] established that the endophytic *Streptomyces* sp. NRRL 30562 obtained from snakevine produces novel peptide antibiotics that possess wide-spectrum of activity against many pathogenic fungi and bacteria. Similar studies [9] with endophytic *Streptomyces* from *Grevillea pteridifolia* also reported the production of antibiotics kakadumycin A and echinomycin effective against Gram-positive bacteria and *Plasmodium falciparum*. Ezra et al. [10] produced coronamycin from *Streptomyces* sp. isolated from *Monstera* sp. that was effective against *P. falciparum* and *Cryptococcus neoformans*. Miller et al. [11] obtained *Pseudomonas viridiflava* that produced an antibiotic ecomycin effective against *Candida neoformans* and *C. albicans*.

Studies on the microbial association with *Ophioglossum* sp. revealed colonization by various species of vesicular arbuscular mycorrhiza (VAM) fungi, like *Endogone microcarpa, Enterophospora* sp.*, Gigaspora* sp.*, Glomus epigaeum, G. macrocarpum,* and *G. occulatum* [12]. Such mycorrhizal association has been reported to improve plant health, disease resistance and drought tolerance. In addition, Nair [13] has also isolated two endophytic fungi, *Fusarium oxysporum* and *F. solani*, from *Ophioglossum* sp. which were found to be non-pathogenic. Septate endophytic fungal associations with roots of *O. reticulatum* are not uncommon. However, bacteria endophytic to *O. reticulatum* have not been reported so far.

The increasing incidence of antibiotic resistance in pathogenic microorganisms in the last few decades raises the demand for finding new alternative antimicrobial agents. In view of this, the aim of this study was primarily to evaluate the antimicrobial activity of the bacterial endophytes isolated from *Ophioglossum reticulatum* L. and to find a suitable strain for production of novel

antimicrobial compound(s) for possible application against pathogenic microorganisms.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

Ophioglossum reticulatum L. (family Ophioglossaceae) plants with healthy leaves and mature spike were collected from Darjeeling hills, West Bengal (27°7′ N and 88°2′ E, 6710′ above sea level) during August-September, 2015-2016. Plants along with soil collected in polythene bags were brought to the laboratory and stored at 4°C until used for the isolation of bacterial endophytes.

2.2 Isolation of Bacterial Endophytes

Bacterial endophytes were isolated from the segments of healthy *O. reticulatum* L. plants following surface sterilization with sodium hypochlorite (0.5%) and ethanol (70%) as per the method described by Sun et al. [14]. Plant segments were aseptically cut into small segments, plated on nutrient agar, glycerol asparagine agar and tryptic soy agar and incubated at 28-30°C for 2-7 days. Morphologically distinguishable bacterial colonies growing out of the plant segments were isolated in pure form by dilution streaking on the same agar media and maintained by regular sub-culturing.

2.3 Characterization and Identification of Endophytes

Morphological and physio-biochemical characterization of endophytes was performed following standard microbiological methods [15]. Antibiotic sensitivity of the bacterial isolates was detected following the Kirby Bauer disc-diffusion assay [16] using antibiotic impregnated discs (Himedia, India, 6 mm dia). The 16S rRNA gene sequence of the strain was determined by direct sequencing of PCR-amplified 16S rDNA. The genomic DNA of the bacterium was isolated and purified following the modified method of Marmur [17] and the 16S rDNA was amplified using the universal primers 8F and 1492R. The PCR amplified product was purified using QIAquick gel extraction kit (Qiagen, Netherlands) and the sequencing reaction was performed with ABI PRISM Dye Terminator cycle-sequencing ready reaction kit (Applied Biosystems). The sequencing products were purified and electrophoresed on polyacrylamide sequencing gel using an ABI 377 automated DNA sequencer. Sequencing data were analyzed by ABI version 3.0.1 b3 software and compared with reference sequences using the NCBI BLASTN programme. Multiple sequence alignments were carried out by using BLOSUM 62 matrix with the program package Clustal-W employing the neighbor-joining algorithm method [18] with MEGA version 7.0.

2.4 Screening of Endophytes for Antimicrobial Activity

The endophytic bacterial isolates were primarily screened for their antimicrobial activity by cross-streak method using *Staphylococcus aureus, Escherichia coli, Pseudomonas cepacia, Alternaria solani,* and *Saccharomyces cerevisiae* as the test organisms. The endophytic isolates were inoculated on nutrient agar plates as a single streak across the centre and incubated at 32°C for 4 days. Freshly grown cultures of the test organisms were inoculated at right angle to the producer endophytic isolates and incubated again at 32°C for 24 h. The length of the inhibition zones of the test organisms were measured to nearest millimeter.

The positive antimicrobial isolates were further subjected to secondary screening. The isolates were grown in liquid medium under continuous shaking and the cell-free culture filtrate after passing through membrane filter was assayed by agar-cup method using the same test organisms. The plates were incubated at 32°C for 24 h and the diameter of inhibition zones were measured to nearest millimeter.

2.5 Growth Associated Production of Antimicrobial Substance

Time course of growth, sporogenesis and production of antimicrobial substance(s) by the selected endophytic isolate OPL 19 was determined in tryptic soy broth. Erlenmeyer flasks (250 ml) containing 50 ml of medium was inoculated with freshly grown culture (0.5 ml) and incubated at 30°C under continuous shaking condition (120 rpm). Samples were withdrawn aseptically at regular time interval and assessed for growth, pH, sporogenesis and antimicrobial activity. Growth was estimated by measuring the optical density of the culture at 540 nm using a Systronics Photoelectric Colorimeter 112. Percentage of sporulating cells was determined

using haemocytometer and phase contrast microscope (Carl Zeiss). The pH of the medium was estimated using Systronics pH meter (μ pH System 361). To determine the antimicrobial activity, the culture was aseptically centrifuged (10,000xg, 4°C, 12 min), the cell-free supernatant was passed through membrane filter and used for agar-cup assay in nutrient agar plates seeded with *S. aureus* and *E. coli*. The plates were incubated at 32°C for 24 h and diameter of inhibition zone was measured to nearest millimeter.

2.6 Isolation and Purification of Antibacterial Compound

The isolate OPL 19 was grown in tryptic soy broth for 102 h under continuous shaking and centrifuged (10,000xg, 4°C, 15 min) to separate the cell-free culture filtrate and the cell mass. The cell-free culture filtrate was extracted individually with butanol, chloroform, ethyl acetate, hexane and benzene for three times. The solvent extracts were pooled, evaporated to dryness under reduced pressure, dissolved in sterile distilled water and used for antibacterial activity by agar-cup assay using *S. aureus* and *E. coli* as test organisms. Attempts were also made to isolate the antibacterial compound(s) if any from washed cell masses using the same solvents.

2.7 Thin Layer Chromatography

Ethyl acetate extract of the culture filtrate was subjected to separation by TLC using a number of solvent systems and visualized under UV light and in iodine vapor to determine the number of compound(s) present. Each of the spots was scrapped out from the plate, eluted with solvent, evaporated to dryness, dissolved in sterile water and assayed in the usual way to determine antibacterial activity.

2.8 Bioautography

For bioautography, the ethyl acetate extract was subjected to paper chromatographic separation with chloroform: methanol (1:3) as the solvent system. The dried chromatogram was cut out in form of narrow strips and was placed on a nutrient agar plate seeded with *S. aureus*. It was removed after soaking for 2 min and the plate was subsequently incubated for 24 h at 37°C. The position of the inhibition zone and its Rf value was determined. Parallel strip of the chromatogram was observed under UV light and the Rf value of the spot was calculated and matched with those of the inhibition zone.

2.9 Determination of the Nature of the Antibacterial Compound

To determine the nature of the antibacterial compound, TLC of the aqueous solution of the ethyl acetate extract was done with chloroform: methanol (1:3) as the solvent system. The plate was then sprayed with different spraying reagents to identify the nature of the antibacterial compound following the methods as described in Stahl [19]. UV-absorption spectrum of the partially purified compound was determined in a Jenway 6505 UV-Vis Spectrophotometer.

3. RESULTS

3.1 Primary Screening of Endophytic Isolates for Antimicrobial Activity

A total of 29 phenotypically distinguishable endophytic bacteria were isolated in pure form from different plant organs of *O. reticulatum* L. collected from Darjeeling hills of West Bengal, India. These isolates were screened primarily for antimicrobial activity by cross-streak method using *S. aureus, P. cepacia, E. coli, A. solani,* and *S. cerevisiae* as test organisms. Only 7 of them showed antibacterial activity against the test organisms (Table 1). The isolates OPL 08, OPL 11, OPL 19, and OPR 07 were able to inhibit all the test bacterial strains, while isolates OPL 17, OPR 03, and OSK 08 could inhibit two of the three bacterial strains. None of the endophytes were able to inhibit the test fungi, *A. solani* and *S. cerevisiae*.

3.2 Secondary Screening for Antimicrobial Activity

These 7 antimicrobial isolates were grown in nutrient broth under continuous shaking for 4 days and the cell-free culture filtrate was assayed against test bacterial and fungal strains by agar-cup assay. Results as shown in Table 2 have indicated the inability of isolates OPL 08 and OSK 08 to produce antibacterial substance(s) in liquid medium. Cell-free culture filtrates of OPL 11, OPL 17 and OPR 07 could inhibit only *E. coli*, while the same of OPR 03 was effective against *S. aureus* and *E. coli*. The isolate OPL 19 was considered as the most potent one in terms of antibacterial activity as it inhibited *S. aureus*, *P. cepacia* and *E. coli*. As usual none of the culture filtrates were able to show antifungal activities.

Table 1. Primary screening of bacterial endophytes isolated from *O. reticulatum* L. for production of antimicrobial substances by cross-streak method

Endophytic bacterial isolate	Length of inhibition zone, mm				
	Test organisms				
	Staphylococcus aureus	***Pseudomonas cepacia***	***Escherichia coli***	***Alternaria solani***	***Saccharomyces cerevisiae***
OPL 08	4.33 ± 0.57	4.66 ± 0.57	3.33 ± 0.57	NI	NI
OPL 11	2.66 ± 0.57	4.00 ± 1.00	4.66 ± 0.57	NI	NI
OPL 17	NI	3.33 ± 0.57	5.66 ± 0.57	NI	NI
OPL 19	11.33 ± 0.57	10.0 ± 1.00	2.66 ± 0.57	NI	NI
OPR 03	7.66 ± 1.00	NI	7.00 ± 1.00	NI	NI
OPR 07	2.33 ± 0.57	3.66 ± 0.57	7.33 ± 0.57	NI	NI
OSK 08	NI	10.0 ± 1.00	7.66 ± 0.57	NI	NI

**NI indicates 'No Inhibition'. Values represent mean of triplicate readings ± S.D*

Table 2. Secondary screening of the selected endophytic bacterial isolates of *O. reticulatum* L. for production of antimicrobial substances following agar-cup assay

Endophytic bacterial isolate	Diameter of inhibition zone, mm				
	Test organisms				
	Staphylococcus aureus	***Pseudomonas cepacia***	***Escherichia coli***	***Alternaria solani***	***Saccharomyces cerevisiae***
OPL 08	NI	NI	NI	NI	NI
OPL 11	NI	NI	8.33 ± 0.57	NI	NI
OPL 17	NI	NI	9.00 ± 1.00	NI	NI
OPL 19	15.0 ± 1.00	10.66 ± 0.57	11.33 ± 0.57	NI	NI
OPR 03	9.33 ± 0.57	NI	9.33 ± 0.57	NI	NI
OPR 07	NI	NI	9.00 ± 1.00	NI	NI
OSK 08	NI	NI	NI	NI	NI

**NI indicates 'No Inhibition'. Values represent mean of triplicate readings ± S.D.*

3.3 Characterization and Identification of Potent Isolate

The potent isolate OPL 19 obtained from leaf tissues of *O. reticulatum* L. was a rod-shaped (Fig. 1), motile, Gram-positive, endospore forming bacterium. It produced a number of enzymes such as catalase, gelatinase, lipase, caseinase, inulinase and could utilize glucose, sucrose, fructose, maltose, lactose, melibiose, raffinose, turanose, inositol as the sole source of carbon.

It also showed resistance to cell wall inhibiting antibiotics ampicillin and penicillin G, but was sensitive to protein synthesis inhibitors streptomycin, chloramphenicol and tetracycline (Table 3).

The 16S rDNA sequence analysis revealed that the isolate *Bacillus* OPL 19 was most closely related to *Bacillus safensis* NBRC 100820 (KY174336.1) with a very high sequence similarity (99%), reasonably high score and e-value being zero. The 16S rDNA sequence of *Bacillus* OPL 19 has been deposited to the GenBank under the accession number KY029081 and designated as *Bacillus safensis* OPL 19. The evolutionary relationship of the isolate OPL 19 as depicted from the dendrogram showed clear rooted evolution (Fig. 2). Phylogenetically, *Bacillus* OPL 19 also showed close relationship with *Bacillus pumilus* ATCC 7061 (GQ911554.1) but differed significantly from *B. pumilus* in terms of carbon source utilization pattern and fermentation (Table 4).

3.4 Growth Associated Antibiotic Production

Media of five different types were evaluated for growth associated production of antibiotic substance and it was observed that growth of *Bacillus* OPL 19 was enhanced in tryptic soy broth followed by casein hydrolysate peptone medium, tryptone commercial sugar medium and nutrient broth, as compared to Gause mineral salts broth and Lindenbein synthetic medium.

Though the growth of the isolate varied significantly with respect to the composition of media, the antibacterial activity of the cell-free culture filtrate of the endophytic *Bacillus* OPL 19 did not show considerable differences (Table 5).

In view of comparatively better growth and antibiotic production, time course of growth and antibiotic production by *Bacillus* OPL 19 was conducted in tryptic soy broth under batch culture. Production of antibiotic was initiated in the mid exponential phase and reached its maximum after 96h of growth during which most of the cells had sporulated (Fig. 3a and 3b).

3.5 Isolation and Partial Purification of the Antibiotic

The antibacterial compound(s) was extracted from the cell-free culture filtrate of *Bacillus* OPL 19 (grown in tryptic soy broth) by butanol, chloroform, ethyl acetate, hexane and benzene. The solvent extracts were evaporated to dryness, dissolved in sterile water and assayed by agar-cup method. Under identical conditions, the ethyl acetate fractions showed highest inhibition although the butanol and chloroform extracts were not inferior when tested against *S. aureus* and *E. coli.* Hexane and benzene appeared to be poor extractants for the antibiotic compound. Cell mass extracted with all the five solvents mentioned above failed to produce any inhibition zone against the test organisms.

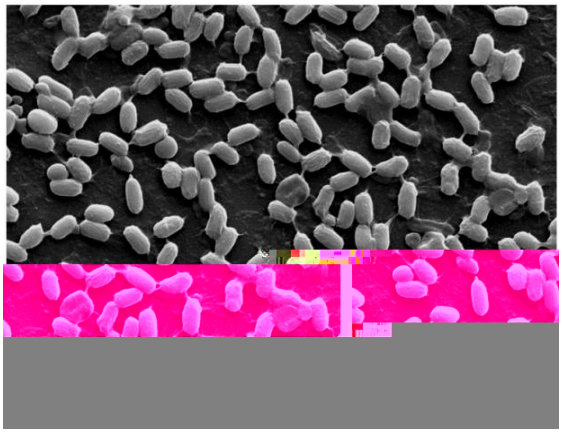

Fig. 1. Scanning electron microscopy showing micromorphology of the endophytic bacterium, *Bacillus* OPL 19 isolated from *O. reticulatum* L.

Table 3. Morphological, physiological and biochemical characteristics of the bacterial isolate OPL 19 endophytic to *O. reticulatum* L.

Character	Response
Colony morphology	Smooth, white opaque with regular margin
Cell morphology	Rods, mostly solitary, 3.0 µm X 0.7 µm
Gram nature	Gram + ve
Motility	+
Production of diffusable pigment	-
Endospore formation	+ (central)
Optimum temperature for growth	32°C
Optimum pH for growth	7.0
Production of catalase	+
Production of gelatinase	+
Production of caseinase	+
Production of amylase	-
Production of lipase	+
Reduction of nitrate	-
Production of inulinase	+
Production of pectinase	-
Production of cellulase	-
Degradation of P(3HB)	-
Utilization of glucose, sucrose, fructose, maltose, lactose, melibiose, raffinose, turanose, inositol	+
Antibiotic sensitivity profile	Amp^r, P^r, C^s, S^s, $S3^s$, TE^s

+ = positive response, - = negative response, 'r' = resistant, 's' = sensitive
Amp = Ampicillin (10 mcg), P = Penicilin G (1U), C = Chloramphenicol (25 mcg), S = Streptomycin (10 mcg), S3 = Sulphatriad (300 mcg), TE = Tetracycline (25 mcg)

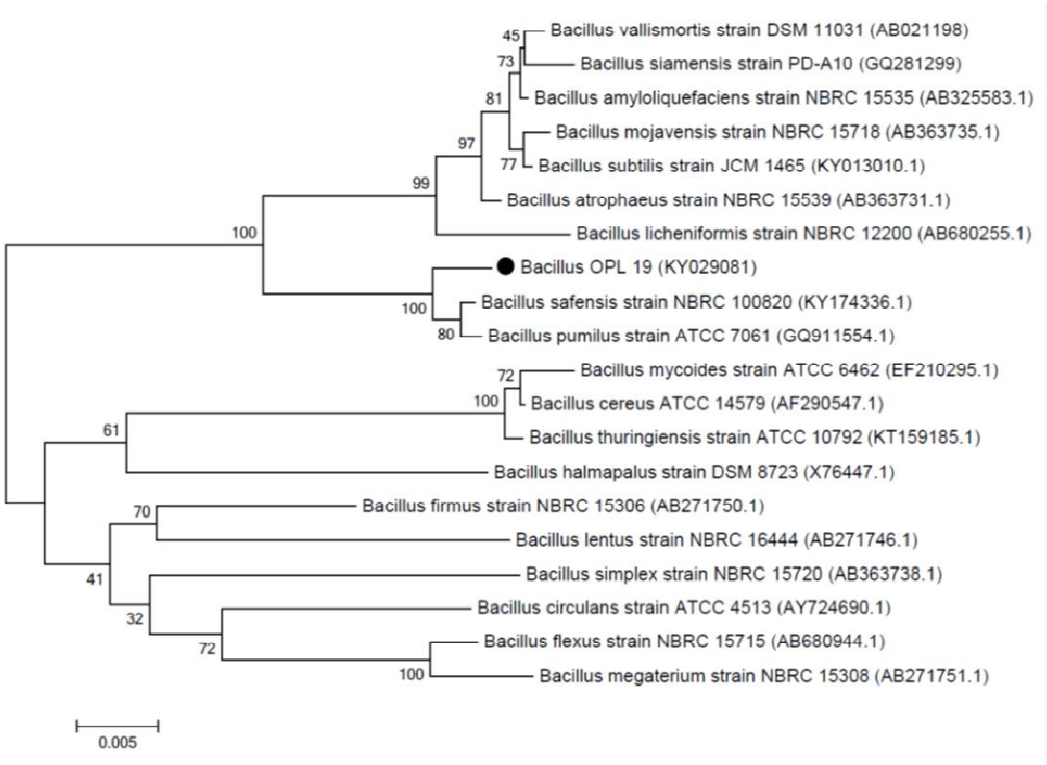

Fig. 2. Phylogenetic relationship of *Bacillus* OPL 19 (GenBank accession number KY029081) with closely allied *Bacillus* spp. based on 16S rDNA sequence analysis

Table 4. Comparison of biochemical characters of *Bacillus* OPL 19 with *Bacillus safensis* NBRC 100820 and *Bacillus pumilus* ATCC 7061

Character	*Bacillus* OPL 19 (KY029081)	*Bacillus safensis* NBRC 100820 (KY174336.1)	*Bacillus pumilus* ATCC 7061 (GQ911554.1)
Enzyme production			
Caseinase[a]	+	-	+
Lipase[a]	+	-	+
Acid production from			
Inositol	+	+	-
Maltose	+	+	-
Turanose	+	+	-
Utilization of			
Inositol	+	+	-
Melibiose	+	+	-
Raffinose	+	+	-
Maltose	+	+	-
Turanose	+	+	-

[a]*Few strains of Bacillus safensis are reported to produce lipase and caseinase although the type strain B. safensis NBRC 100820 (KY174336.1) gave negative reaction [20]*

The ethyl acetate extract of the culture filtrate of *Bacillus* OPL 19 was subjected to thin layer chromatography (TLC) using a number of different solvent systems, each of which indicated the presence of a single spot with different Rf values which ranged from 0.14 – 0.9 (Table 6) as visualized by UV light and iodine vapour. The compound corresponding to the single spot in chloroform: methanol (1:3) mixture was separated by preparative TLC and its

homogeneity was further confirmed by TLC. The antibacterial activity of the homogenous compound was evaluated by bioautographic method following paper chromatography in chloroform: methanol (1:3) mixture. The paper strip produced a single inhibition zone when tested on *S. aureus* seeded nutrient agar plate. The Rf of the inhibition zone corresponds to the Rf value of the single spot detected in parallel paper strip following exposure to UV light and iodine vapour.

3.6 Nature of the Antibacterial Compound

The antibiotically active ethyl acetate extract of the cell-free culture filtrate of *Bacillus* OPL 19 was developed in TLC using chloroform: methanol (1:3) as the solvent system. The spot so obtained was sprayed with different spraying reagents according to Stahl [19] to identify the nature of the antibacterial compound. Of the nine different detecting reagents used, the antibiotically active spot showed positive response only against bromothymol blue indicating its lipoidal nature, although, the negative response in orcinol test indicated the absence of glycolipid.

The aqueous solution of the partially purified antibiotic elaborated by *Bacillus* OPL 19 was subjected to varying temperatures for 10 min and its antibacterial activity was checked in the usual way by agar-cup assay. The antibacterial compound was quite thermostable as there was no loss of antibiotic activity till 60°C, while at 121°C (autoclaving) it could retain more than 70% of its activity (Fig. 4). The UV-absorption spectra of the partially purified colourless antibiotic of *Bacillus* OPL 19 also showed major peaks at 220 and 235 nm (Fig. 5).

Table 5. Effect of different media on growth and antibiotic production by the endophytic bacterium *Bacillus* OPL 19

Medium used	Growth, O. D. at 540 nm	Final pH of medium	Diameter of inhibition zone, mm	
			Test organisms	
			S. aureus	*E. coli*
Tryptic soy broth	2.19 ± 0.05	8.80 ± 0.04	17.66 ± 0.57	11.33 ± 0.57
Casein hydrolysate peptone medium	1.68 ± 0.06	9.00 ± 0.05	14.00 ± 1.00	12.66 ± 0.57
Nutrient broth	1.46 ± 0.04	8.50 ± 0.08	16.00 ± 1.00	12.66 ± 0.57
Tryptone commercial sugar medium	1.61 ± 0.08	6.00 ± 0.02	16.33 ± 0.57	10.00 ± 1.00
Gause mineral salts medium	1.17 ± 0.07	6.50 ± 0.34	15.00 ± 1.00	11.00 ± 1.00
Lindenbien synthetic medium	1.13 ± 0.08	6.00 ± 0.04	15.00 ± 1.00	10.66 ± 0.57

**Antibacterial activity of the cell-free culture filtrate was assessed following agar-cup assay method using S. aureus and E. coli as test organisms. Values represent mean of triplicate readings ± S.D*

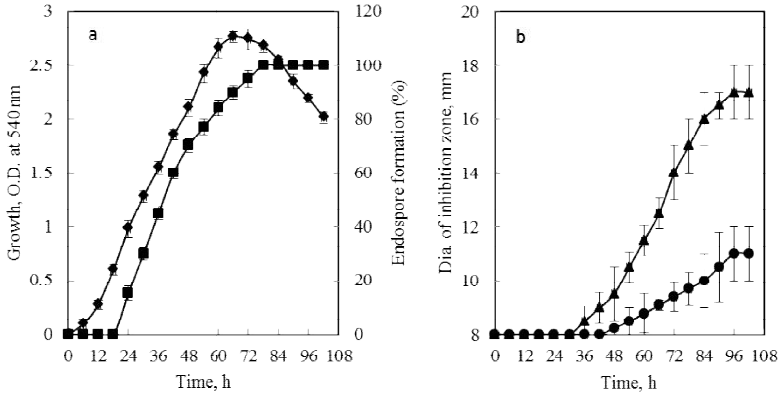

Fig. 3. Time course of growth (◆), endospore formation (■) [3a] and antibiotic production by the endophytic bacterium *Bacillus* OPL 19 [3b] isolated from *O. reticulatum* L. as determined against *S. aureus* (▲) and *E. coli* (●)

[Bacillus OPL 19 was grown in tryptic soy broth under continuous shaking, samples withdrawn at every 6 h interval were evaluated for growth (O.D. at 540 nm) and antibiotic production (agar-cup assay against S. aureus and E. coli) and the endospores formed were counted by a haemocytometer under phase contrast microscope]

Table 6. Thin layer chromatographic separation of the ethyl acetate extract of the culture filtrate of the endophytic bacterial isolate *Bacillus* OPL 19

Solvent systems	Rf
Chloroform: Methanol (1:3)	0.90
Butanol: Acetic acid: Water (4:1:1)	0.70
Benzene: Ethyl acetate (1:9)	0.15
Acetone: Water (1:1)	0.84
Ethyl acetate: Methanol: Water (40:5:5)	0.39
Chloroform: Ethanol: Water (50:50:8)	0.87
Butanol: Ethanol: Water (3:3:2)	0.72
Benzene saturated with water	0.14
Butanol: Methanol: Water (4:1:5)	0.71

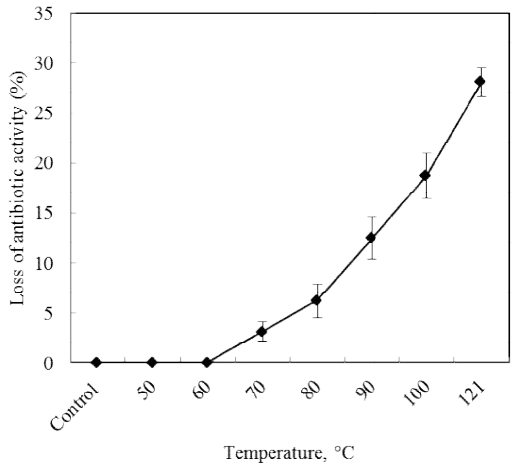

Fig. 4.Thermostability of the antibacterial compound produced by the endophytic bacterium *Bacillus* OPL 19 isolated from *O. reticulatum* L.

[The loss of antibiotic activity of the heat-treated partially purified compound produced by Bacillus OPL 19 was determined by agar-cup assay against S. aureus]

3.7 Antimicrobial Spectrum of the Antibiotic

The aqueous solution of the partially purified antibiotic elaborated by *Bacillus* OPL 19 was finally tested against different bacterial and fungal test organisms by agar-cup assay and the antimicrobial spectrum is shown in Table 7. The results indicated that the antibiotic of *Bacillus* OPL 19 was antibacterial in nature and was able to inhibit a large number of Gram-positive and Gram-negative bacteria excluding *Salmonella typhimurium.* However, as usual it failed to produce any antifungal activity against the tested organisms.

Table 7. Antimicrobial spectrum of the partially purified antibacterial antibiotic produced by the endophytic *Bacillus* OPL 19

Test organism	Diameter of inhibition zone, mm[a]
Bacteria	
Acinetobacter baumannii	13.33 ± 0.57
Arthrobacter sp.	10.66 ± 0.57
Bacillus cereus	10.00 ± 1.00
Bacillus subtilis	13.00 ± 1.00
Cellulosimicrobium cellulans	11.66 ± 0.57
Escherichia coli	11.00 ± 1.00
Klebsiella pneumonia	10.00 ± 1.00
Proteus vulgaris	11.33 ± 0.57
Pseudomonas aeruginosa	12.00 ± 1.00
Pseudomonas cepacia	11.33 ± 0.57
Salmonella typhimurium	NI
Staphylococcus aureus	15.00 ± 1.00
Staphylococcus epidermidis	9.66 ± 0.57
Staphylococcus haemolyticus	10.66 ± 0.57
Fungi	
Alternaria solani	NI
Aspergillus niger	NI
Curvularia sp.	NI
Penicillium citrinum	NI
Saccharomyces cerevisiae	NI

[a]*The partially purified antibacterial antibiotic of Bacillus OPL 19 was used for antimicrobial assay by agar-cup method using nutrient agar for bacteria, Czapek-dox agar for filamentous fungi and Sabaourd's dextrose agar for S. cerevisiae. NI indicates 'No Inhibition'. Values represent mean of triplicate readings ± S.D.*

4. DISCUSSION

Antimicrobial substances are widely produced by bacteria of the genus *Bacillus* and include subtilin, [21], subtilosin [22], coagulin [23], and megacin [24]. This study revealed that *Bacillus* OPL 19 isolated from *Ophioglossum reticulatum* L. was the best producer of antimicrobial substances from among the 29 evaluated endophytic isolates. Screening experiments (Table 1) revealed that out of the seven isolates which gave positive results in cross-streak method, only five showed production of antimicrobial substances in the liquid media as evaluated by agar-cup assay. None of the isolates were antifungal (Table 2). The negative results in agar-cup assay might be due to the inability of these isolates in producing the antibacterial compound in liquid culture.

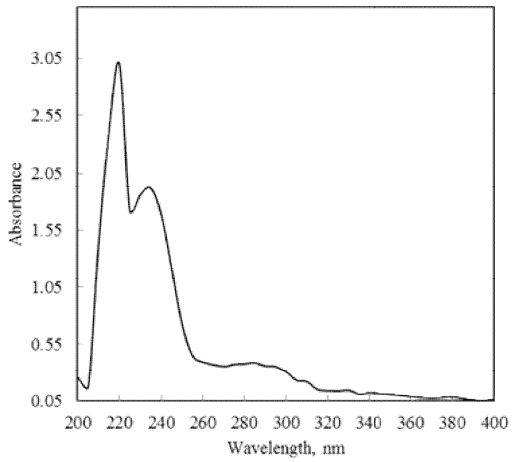

Fig. 5. UV-absorption spectra of the partially purified antibacterial compound produced by the endophytic bacterium *Bacillus* OPL 19 isolated from *O. reticulatum* L.

[The antibiotic extracted in ethyl acetate was partially purified by preparative TLC in chloroform: methanol (1:3) mixture. Absorption spectrum of the aqueous solution of the antibiotic was determined in the range of 200-400 nm]

The endophytic isolate *Bacillus* OPL 19 was identified as *Bacillus safensis* based on morphological (Fig. 1), physio-biochemical (Table 3) and 16S rDNA sequence analysis (Fig. 2). It showed a very high sequence similarity (99%) with *Bacillus safensis* strain NBRC 100820 as well as with *Bacillus pumilus* strain ATCC 7061. *Bacillus safensis* OPL 19, however, differed from *B. pumilus* in its ability to utilize inositol, melibiose, raffinose, maltose, turanose and acid production from inositol, maltose and turanose (Table 4).

Several strains of *B. safensis* have been reported to produce antifungal, antibacterial and antiviral effects [25,26] and were strongly antagonistic to pathogenic *Pseudoalteromonas* sp. and *Pseudoalteromonas tetraodonis* [27]. *B. safensis* has also been used as a biocontrol agent against Oomycetous plant pathogens [28], causative agent of tomato grey mould and harmful cyanobacterium, *Microcystis aeruginosa* [29]. *B. safensis* HA-MS-105 isolated from the sponge *Amphimedon ochracea*, has potential cytotoxicity against HepG2 (hepatocellular carcinoma), HCT (colon carcinoma) and MCF-7 (breast carcinoma) cancer cell lines [30].

Bacillus OPL 19 was able to produce the antibiotic substance in a variety of media differing in composition (Table 5) and the best production was evident in tryptic soy broth (Fig. 3) under batch culture. The antibiotic compound so produced was extracted from the cell-free culture filtrate of *Bacillus* OPL 19 with ethyl acetate and the homogeneity of the compound was confirmed by developing with various solvent systems (Table 6) and detection with UV light, iodine vapour and bioautography. The partially purified compound showed broad spectrum of antibacterial activity being effective against a wide range of both Gram-positive and Gram-negative bacteria (Table 7). The antibacterial substance was thermostable (Fig. 4), relatively non-polar and lipoidal in nature. Reports of lipid antimicrobials, bacilysocin, produced by *Bacillus subtilis* effective against bacteria and fungi are not uncommon [31]. Similarly, Shinde et al. [32] also isolated a phospholipid antimicrobial compound from acidophilic *Bacillus subtilis* with broad spectrum of activity. UV-absorption spectrum of the partially purified antibacterial compound of *Bacillus* OPL 19 showed major peaks at 220 and 235 nm (Fig. 5) very close (225 and 232 nm) to those of oligomycin A [33]. Antimicrobial compound extracted from *Streptomyces* sp. also showed UV absorbance at 226 and 246 nm [34]. The ultraviolet absorbance spectrum of lipopeptide antimicrobials produced by *Bacillus* sp. however showed maximum absorbance at 235 nm [35], while antibiotics like tetracycline and showdomycin have distinct absorbance only at 220 nm [36,37].

Finally, it may be mentioned that though during present preliminary study we could not have determined the identity of the antibacterial compound produced by *Bacillus safensis* OPL 19 endophytic to *Ophioglossum reticulatum* L., further chemical analysis may help us to determine the chemical nature of the compound. Such in depth analysis of the antibacterial metabolite elaborated by endophytic bacteria may be of use in pharmacological and biotechnological purposes.

5. CONCLUSION

Ophioglossum reticulatum L. is a medicinal fern used traditionally as an anti-inflammatory medicine. It harbors many endophytic bacteria with antibacterial activity. *Bacillus safensis* is one such species which produced a broad spectrum thermostable antibiotic capable of inhibiting both Gram-positive and Gram-negative bacteria. Our present study concludes that the endophytic bacteria of *Ophioglossum reticulatum* L. could be a potential bioresource for discovery of novel antibacterial antibiotics.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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