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Proteolytic Activity of *Bacillus amyloliquefaciens* UEF01 Endophytic to Carnivorous Plant *Utricularia exoleta* R. Br.

Madhubanti Chaudhuri[1], Sayantani Biswas[1], Arundhati Pal[2] and A. K. Paul[1*]

[1]Laboratory of Microbiology, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700 019, India.
[2]Department of Botany, Serampore College, 9, William Carey Road, Serampore, Hooghly, West Bengal 712201, India.

Authors' contributions

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

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ABSTRACT

Aims: The endosphere of the carnivorous plant *Utricularia exoleta* R. Br. represents a unique niche for the study of microbial diversity. This study was aimed at to isolate and enumerate the protease producing potential of bacteria endophytic to *U. exoleta* R. Br.
Study Design: Extracellular proteolytic activity of the cell-free culture filtrate of the bacterial endophyte was determined following standard colorimetic assay using casein as the substrate.
Place and Duration of Study: The study was undertaken in the Microbiology Laboratory, Department of Botany, University of Calcutta during September 2015 and March 2016.
Methodology: A total of 36 phenotypically distinguishable bacteria endophytic to leaf, stem, bladder and fruit of *U. exoleta* were isolated and evaluated for protease production. The best protease producing isolate, UEF01 was selected, characterized and the conditions for protease activity were optimized.

**Corresponding author: E-mail: amalk_paul@yahoo.co.in;*
Co-author E-mail: madhubanti.chaudhuri@gmail.com;

Results: The selected isolate was characterized following morphological, physio-biochemical and 16S rRNA gene sequence analysis and identified as *Bacillus amyloliquefaciens* UEF01 (GenBank Accession No. KX816572). The crude protease of the cell-free culture filtrate of UEF01 was maximum at 35°C, pH 9 with 1.5% (w/v) casein. The enzyme appeared to be thermolabile with loss of >80% activity at 70°C (15 min incubation). Kinetic studies indicated the K_m and V_{max} values as 9.21 mg/ml and 71.43 U/mg of protein respectively. The enzyme was sensitive to Na and Mn ions as well as some selective protease inhibitors such as phenyl methyl sulfonyl fluoride, β-mercaptoethanol, and EDTA.
Conclusion: This proteolytic study will help in understanding the role of endophytes in digestion of prey within the bladders of the carnivorous plants.

Keywords: Carnivorous plants; bacterial endophytes; Utricularia exoleta; protease; enzyme kinetics; Bacillus amyloliquefaciens.

1. INTRODUCTION

Utricularia exoleta R. Br., commonly known as bladderwort is an aquatic free-floating carnivorous herb belonging to the family Lentibulariaceae and grows in shallow water and swamps deficient in phosphorus and nitrogen. Nutritional requirements of the plant are fulfilled by capturing and digesting small aquatic invertebrates in their modified-leaves or bladders. The bladders contain sensitive bristles towards their mouth and are closed by trap doors that open inwards [1]. The prey when comes in contact with the bristles, the door opens carrying it into the bladder along with water current where it is digested by the hydrolytic enzymes of the plant itself and possibly with those of the endophytic microorganisms inhabiting the bladder [2].

The culture-dependent studies of endophytes from carnivorous plants [2,3,4,5,6] and their involvement in the prey decomposition, nutrient mineralization and fixation as well as stress tolerance of the host have long been hypothesized and speculated [2]. So far, the digestion of prey in the traps, bladders or pitchers is concerned; the endophytes are likely to play an important role especially in those carnivorous species which do not produce hydrolytic enzymes of their own. *In vitro* production of hydrolytic enzymes by pitcher fluid bacteria of *Nepenthes* spp. [7,8], *Drosera* [9] and *Utricularia* traps [10] have been documented. However, reports on the occurrence and diversity of endophytes in *Utricularia* spp. and their exploration for production of bioactive compounds are inadequate.

During the course of screening of bacteria endophytic to *Utricularia exoleta* R. Br. for the production of proteolytic enzyme, a potent protease producing bacterium, *Bacillus amyloliquefaciens* UEF01 was isolated. Here an attempt has been made to determine the optimum conditions for the activity of the enzyme using the cell-free culture filtrate of *B. amyloliquefaciens* UEF01 as the crude extracellular protease.

2. MATERIALS AND METHODS

2.1 Isolation of Endophytic Bacteria

Endophytic bacteria were isolated from leaf, stem, bladder and fruit of healthy, flowering *Utricularia exoleta* R. Br. (Lentibulariaceae) plants collected from the district of Midnapore (22.42°N, 87.31°E), West Bengal, India. The collected plants were washed thoroughly with tap water followed by surface sterilization in 0.05% sodium hypochlorite and 70% ethanol [11]. The surface sterilized samples were washed in sterile distilled water, aseptically cut into small segments and placed on Petri plates containing nutrient agar, tryptic soy agar and Lindenbein synthetic agar. Plates were incubated at 32°C for 2-4 days and observed for growth of bacterial colonies associated with the plant segments. Pure cultures of the morphologically distinguishable bacterial endophytes were developed following dilution-streaking and maintained on the same media.

2.2 Screening of Endophytes for Proteolytic Activity

The endophytic bacterial isolates were inoculated in form of streaks on nutrient agar medium supplemented with 1% (w/v) casein and incubated for 48 h at 32°C. The plates were flooded with protein precipitating reagent and the

proteolytic activity was detected by the formation of clear zone surrounding the growth [12]. Protease index was calculated as the ratio of the width of clear zone including bacterial growth with that of the width of bacterial growth only and the most potent isolate was selected.

2.3 Characterization and Identification of the Potent Endophyte

The selected endophytic bacterium was characterized morphologically and physio-biochemically following standard microbiological methods [12,13]. Antibiotic sensitivity of the endophyte was determined following the Kirby Bauer disc-diffusion method [14] using antibiotic impregnated discs (HiMedia 6 mm dia.).

For 16S rRNA gene sequence analysis, chromosomal DNA of the selected endophytic bacterium was isolated and purified according to the modified method of Murmur [15]. PCR amplification was done using two universal primer sets 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'). The PCR amplified product was purified and subjected to sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was performed using BDT v3.1 Cycle sequencing kit on ABI 3730 xl Genetic Analyzer. Consensus sequence of 16S rDNA was generated and compared with closely related neighbour sequences retrieved from the NCBI database using BLAST search. Phylogenetic analysis was performed by using the software package MEGA6 after obtaining multiple alignments of the data available from the public databases by ClustalW [16]. Bootstrap analysis was made using 1000 replicates.

2.4 Production of Protease by the Selected Isolate

Growth associated production of protease by the selected endophytic isolate was determined in casein supplemented nutrient broth. Erlenmeyer flasks (250 ml) containing 50 ml medium was inoculated with 0.5 ml of overnight grown culture and incubated at 32°C under continuous shaking (120 rpm). Samples were withdrawn at regular interval for determination of growth and protease activity. Growth was measured by estimating the optical density at 540 nm. Protease activity of the cell-free culture filtrate was assayed following the method of Anson [17] and Folin Ciocalteau [18].

2.5 Assay of Protease

An aliquot (500 µl) of 1% (w/v) casein in 50 mM phosphate buffer (pH 7.0) was added to 200 µl of the cell-free culture filtrate and incubated at 40°C for 20 min. The reaction was terminated by addition of 1 ml 10% (w/v) trichloroacetic acid (TCA) and kept at room temperature for 15 min. The unreacted substrate was separated by centrifugation at 10,000Xg for 10 min at 4°C. One ml of the supernatant was added to 4.5 ml of Lowry reagent and incubated at room temperature for 10 min. To this reaction mixture 0.5 ml Folin-Ciocalteau reagent was added and incubated for 30 min in dark. The absorbance was measured at 660 nm [19]. Tyrosine was used as the standard. One unit (U) of protease was defined as the amount of enzyme that releases 1 µg of tyrosine per ml per min [20].

Total protein of the cell-free culture filtrate was estimated following the method of Lowry et al. [19] using bovine serum albumin (BSA) as the standard. Specific activity of the protease was obtained by dividing the total protease activity with the amount of protein in the cell-free culture filtrate and expressed as U/mg of protein.

2.6 Statistical Analysis

All the experiments were performed in triplicates and the data shown represent the mean value ± standard deviation.

3. RESULTS

3.1 Isolation of Endophytic Bacteria and Screening for Protease Activity

A total of 36 phenotypically distinguishable bacteria were isolated from 209 segments of surface-sterilized leaf, stem, bladder and fruit of *U. exoleta* R. Br. incubated on nutrient agar, tryptic soy agar and Lindenbein synthetic agar for 48-96 h at 32°C. The isolates were qualitatively and quantitatively screened for protease production on casein supplemented nutrient agar and nutrient broth. The isolate UEF01, endophytic to *U. exoleta* fruit, showing the highest protease index (2.33) (Fig. 1) and specific activity (35.98 U/mg) after 96 and 24 h of growth on casein supplemented nutrient agar and broth respectively was selected for detailed characterization, taxonomic identification and determination of conditions for the protease activity.

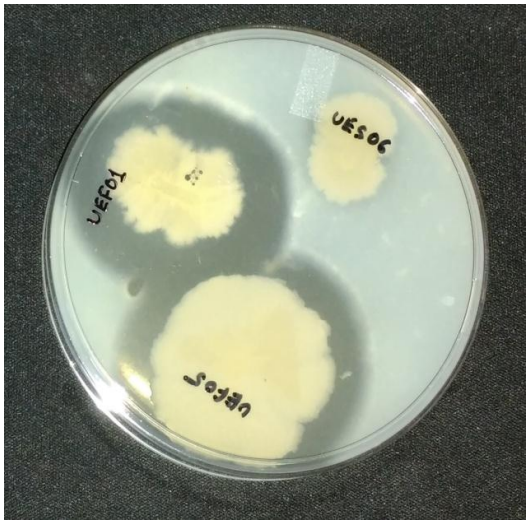

Fig. 1. Photomicrograph showing the proteolytic activity of the endophytic bacterial isolates on casein supplemented nutrient agar medium

3.2 Characterization and Identification of the Bacterial Isolate UEF01

The morphological, physiological and biochemical characteristics of the selected bacterial endophyte UEF01 as determined by standard microbiological methods are shown in Table 1. The Gram-positive, sporulating, motile rod-shaped bacterium produced catalase, oxidase, amylase, gelatinase and pectinase, but could not produce urease, nitrate reductase, cellulase and lipase. The isolate grows well in MacConkey agar, King's A and B agar and also accumulated poly-β-hydroxybutyrate as intracellular inclusion bodies. Antibiotic sensitivity pattern of the isolate indicated its sensitivity to a number of antibiotics, but was resistant mainly to cell wall inhibitors such as penicillin G, methicillin and bacitracin (Table 1).

3.3 Molecular Characterization of the Selected Endophyte

The 16S rRNA gene sequence (1376 bp) of the isolate UEF01 was compared with the reference sequences available in the NCBI databases and the isolate UEF01 was found to have 99% similarity with *Bacillus amyloliquefaciens* (GenBank Accession No. KX816572) (Fig. 2). The isolate was designated as *Bacillus amyloliquefaciens* UEF01 and also showed close relationships with *B. atrophaeus* (99%), *B. subtilis* (99%) and *B. licheniformis* (98%).

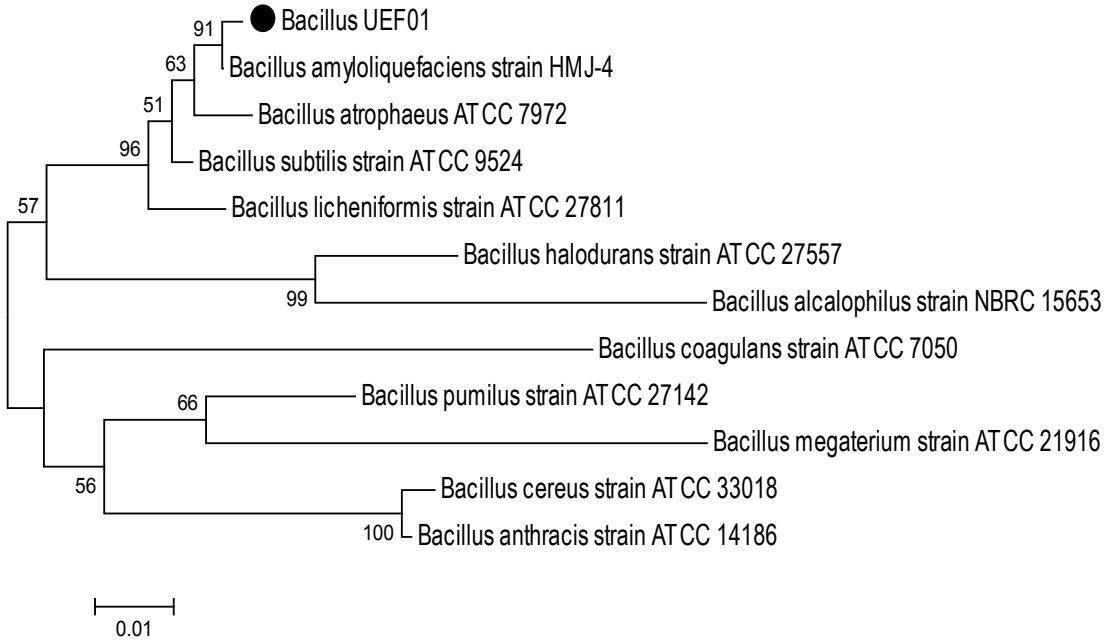

Fig. 2. Phylogenetic relationship of the endophytic bacterial isolate UEF01 with other closely related species based on 16S rRNA gene sequence analysis

Table 1. Morphological and physio-biochemical characters of the endophytic bacterial isolate UEF01

Character	Response	Character	Response	
Colony morphology	White with wrinkled surface	Fermentation and utilization of	Fermentation	Utilisation
Cell morphology	Rods, 2.5-3 X 0.6-1.25 µm	Glucose	+	+
Gram nature	Gram +ve	Maltose	+	+
Motility	+	Sucrose	+	+
Endospore formation	+	Mannitol	+	+
Production of catalase	+	Sorbitol	+	+
oxidase	+	Fructose	-	+
amylase	+	Galactose	-	+
gelatinase	+	Lactose	-	+
pectinase	+	Rhamnose	-	-
urease	-	Raffinose	-	+
nitrate reductase	-	Arabinose	-	+
cellulase	-	Aldonitol	-	-
lipase	-	Dulcitol	-	-
Indole production	-	Trehalose	-	+
Citrate utilization	-	Inositol	-	-
PHB production	+	Mannose	-	+
Growth on MacConkey agar, King's A and King's B agar	+	Antibiotic susceptibility	Met^r, Pen^r, Bac^r, Amp^s, Cip^s, Rif^s, Gen^s, Tri^s, Van^s, Tet^s, Kan^s, Ct^s, C^s	

Met= methicillin, Pen= penicillin G, Bac= bacitracin, Amp= ampicillin, Cip= ciprofloxacin, Rif= rifampicin, Gen= gentamycin, Tri= trimethoprim, Van= vancomycin, Tet= tetracycline, Kan= kanamycin, Ct= chlortetracycline, C= chloramphenicol,; ^{s}= sensitive, ^{r}= resistant

+ indicates positive response, - indicates negative response

3.4 Production of Protease by *B. amyloliquefaciens* UEF01

Growth associated protease production by UEF01 was monitored in nutrient broth supplemented with 1% casein under batch cultivation. Results show that production of protease was more or less parallel with growth of the organism, which attained a peak (35.98 U/mg of protein) during the early stationary phase (24 h) of growth. Following this, there was a significant decrease in production of protease and the medium turned alkaline during the subsequent phases of growth (Fig. 3).

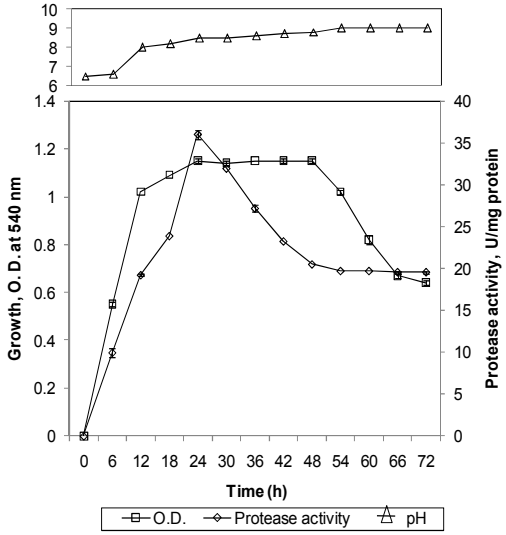

Fig. 3. Time course of growth and production of proteolytic enzyme by *B. amyloliquefaciens* UEF01 in casein supplemented nutrient broth

1 U of protease = amount of enzyme that releases 1 µg of tyrosine per ml per min, values represent mean of triplicate readings ± SD

3.5 Optimization of Conditions for Protease Activity

To determine the optimum conditions for protease activity, the cell-free culture filtrate of the bacterial isolate UEF01 harvested at the early stationery phase (24 h incubation) of growth was used as the source of the crude extracellular enzyme. Effect of a number of factors such as temperature, pH, substrate concentration, metal ions and inhibitors were tested on the activity of the crude protease.

3.5.1 Effect of temperature and pH

The effect of temperature on the protease activity of the cell-free culture filtrate of UEF01 was estimated in the range of 20°C-70°C. The crude enzyme showed its highest activity at 35°C following which it declined sharply with increase in temperature. About 80% of the protease activity was lost at 70°C (Fig. 4A).The effect of pH on the crude protease of UEF01 was estimated in the range of pH 5.0-10.6 using citrate (pH 5-6.2); phosphate (pH 6.2-7.8); Tris-HCl (pH 7.8-8.6) and Glycine-NaOH (pH 8.0-10.6) buffers. Maximum protease activity was obtained at pH 9.0 (55.69 U/mg of protein) (Fig. 4B).

3.5.2 Thermostability of the protease

To test the thermostability of protease, the cell-free culture filtrate of UEF01 was subjected to temperatures ranging from 40°C- 70°C for 15 min. The enzyme lost about 63 and 80% of its activity when the cell-free culture filtrate of UEF01 was subjected to 60°C and 70°C respectively for 15 min. However, incubation of the culture filtrate for 5 min in the tested temperature range hardly affected the activity of the protease of UEF01 (Fig. 5).

3.5.3 Effect of substrate concentration

The protease activity of the cell-free culture filtrate of bacterium UEF01 was found to increase with increasing concentration of casein in the reaction mixture and the maximum enzyme activity (38.9 U/mg of protein) was observed at 1.5% (w/v) casein, beyond which enzyme activity did not increase further (Fig. 6A). The enzyme kinetics followed a linearized Lineweaver-Burk plot (Fig. 6B) and the K_m and V_{max} of the enzyme were calculated as 9.21 mg/ml and 71.43 U/mg protein respectively.

3.5.4 Effect of metal ions

Presence of different metal ions like Na, Ca, Mg, Fe, Co, Cd, Mn, Cu and Ba (0.5-2 mM) as chloride salts inhibited the protease activity UEF01 (Fig. 7). At the highest concentration (2 mM), Mn was most inhibitory (77%), while Cu appeared to be least inhibitory (23.77%) to the crude protease. According to their degree of inhibition on the activity of the protease, the metals could be arranged in the following order Mn>Na>Ba>Co>Ca>Fe>Cd>Mg>Cu.

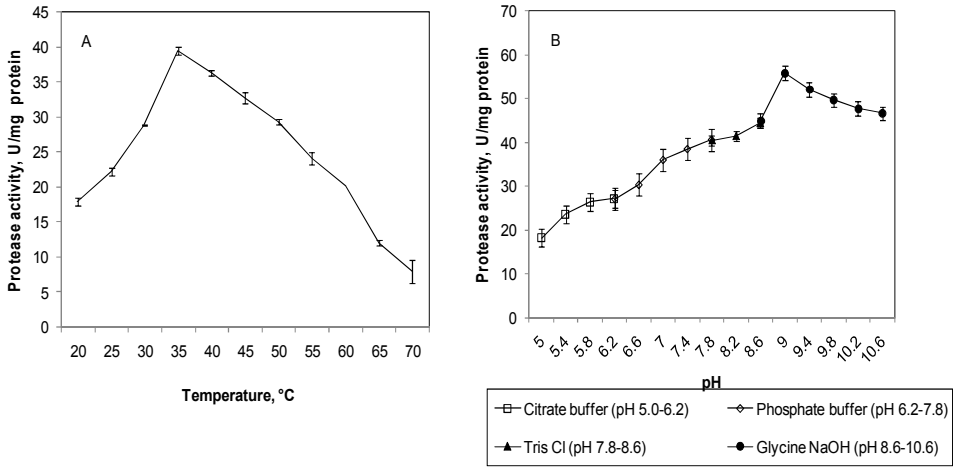

Fig. 4. Effect of temperature (A) and pH (B) on protease activity of the cell-free culture filtrate of B. *amyloliquefaciens* UEF01

1 U of protease = amount of enzyme that releases 1 µg of tyrosine per ml per min, values represent mean of triplicate readings ± SD

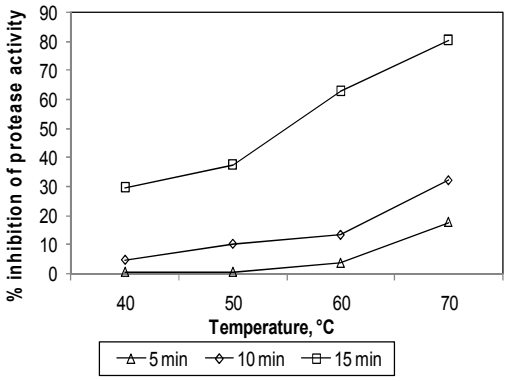

Fig. 5. Thermostability of the crude protease in cell-free culture filtrate of *B. amyloliquefaciens* UEF01

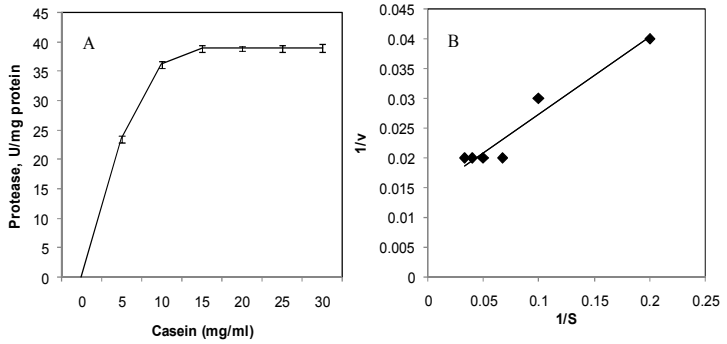

Fig. 6. Effect of substrate concentration on the activity of protease produced by *B. amyloliquefaciens* UEF01 (A) and linearized Lineweaver-Burk plot for enzyme activity (B)

1 U of protease = amount of enzyme that releases 1 µg of tyrosine per ml per min, values represent mean of triplicate readings ± SD

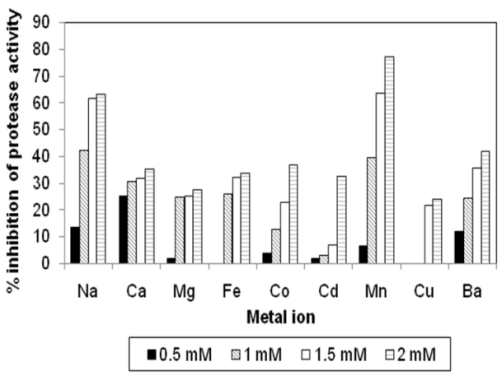

Fig. 7. Effect of metal ions on the activity of the crude protease produced by *B. amyloliquefaciens* UEF01

3.5.5 Effect of inhibitors

The presence of ethylene diaminetetraacetic acid disodium salt (EDTA), β-mercaptoethanol, phenyl methyl sulfonyl fluoride (PMSF) and 1,10-phenanthroline inhibited the activity of protease produced by the isolate *B. amyloliquefaciens* UEF01 (Table 2). The inhibitory effect increased with increasing concentration of the chemicals. While the maximum inhibition (49.23%) was observed at 10 mM of PMSF, the presence of EDTA and β-mercaptoethanol showed moderate inhibition with retention of >65% protease activity. However, nearly 90% enzyme activity was retained in presence of 10 mM of 1, 10-phenanthroline.

4. DISCUSSION

The culturable diversity of bacteria including the diazotrophic and endophytic ones associated with a variety of carnivorous plants has been studied in the recent past. Nitrogen- fixing bacteria belonging to the genera *Bacillus, Burkholderia, Methylobacterium, Paenibacillus, Pseudomonas* and *Sphingomonas* were isolated from the interior of the roots and leaves of *Drosera villosa* var. *villosa* [3], while majority of the endophytic bacteria reported from the medicinally important *Nepenthes* spp. belonged to the genus *Bacillus* [21]. In addition, bacterial communities associated with the pitcher and pitcher fluids of *Sarracenia purpurea* [22,23], *S. alata* [24], *S. minor* [25] along with those of *Nepenthes* spp. [26] have been explored. Likewise, studies with traps of *Utricularia vulgaris*, *U. foliosa* and *U. purpurea* have revealed that more than 58% of their microbial biomass is comprised of bacteria [10] and the microbial communities residing in the traps of *U. hydrocarpa* were dominated by *Aeromonas* and *Acidomonas* spp. [27]. The present culture-dependent study revealed that the internal tissues of leaf, stem, bladder and fruit of *U. exoleta* R. Br. also harbour a wide diversity of phenotypically distinguishable cultural bacteria.

Table 2. Effect of different inhibitors on the activity of the protease produced by *B. amyloliquefaciens* UEF01

Inhibitors	Concentration (mM)	Protease activity (U/mg protein)[a]	% inhibition
Control	**-**	**35.57±0.82**	**0**
Ethylene diamine tetraacetic acid	1	34.18±0.27	3.91
	2	34.13±0.54	4.05
	5	31.08±1.09	16.62
	10	24.04±0.41	32.42
β-mercaptoethanol	1	34.92±1.83	1.83
	2	30.90±1.25	13.13
	5	24.29±0.32	31.71
	10	23.43±0.94	34.13
Phenyl methyl sulfonyl fluoride	1	33.21±2.88	3.64
	2	29.56±0.70	16.90
	5	25.61±1.93	28.01
	10	18.06±2.83	49.23
1,10-phenanthroline	1	34.67±0	2.53
	2	34.21±0.70	3.82
	5	33.03±0.13	7.14
	10	31.96±0.14	10.15

1 U of protease = amount of enzyme that releases 1µg of tyrosine per ml per min, [a] values represent mean of triplicate readings ± SD

Reports on the production of hydrolytic enzymes such as cellulase, chitinase, lipase and asparaginase by the endophytic fungi of *Nepenthes* spp. are not uncommon [6], but production of such enzymes by bacteria endophytic to *Utricularia* spp. are scanty. Sirova et al. [10] in their studies on microbial community development in the traps of *Utricularia* spp. documented the extracellular phosphatase activity of the trap associated microorganisms following the technique of epifluorescence microscopy. Bacterial endophytes of *U. exoleta* isolated (36) in this study have been screened and majority of them were found to produce the extracellular proteases. The isolate UEF01 was the most potent one based on its performance in both solid (Fig. 1) as well as liquid media. These proteolytic endophytes could be of importance in contributing to the digestion of insect prey by the host plant.

The endophytic bacterial isolate UEF01, a Gram-positive, endospore forming motile rod, was identified as *Bacillus amyloliquefaciens* UEF01 (GenBank Accession No. KX816572) based on morphological, physio-biochemical (Table 1), and molecular (Fig. 2) characteristics. *B. amyloliquefaciens* has been reported in vanilla orchids and their cultivated hybrids as a systemic endophyte [28], in ornamental hosta [29] and also in *Ophiopogon japonicus* [30]. Time course studies on growth and protease production by *B. amyloliquefaciens* UEF01 revealed maximum extracellular protease production in the cell-free culture filtrate after 24 h of incubation (Fig. 3). The protease activity of the cell-free culture filtrate of *B. amyloliquefaciens* UEF01 has been optimized with respect to temperature and pH (Fig. 4A & B) and appeared to be more or less similar to alkaline proteases reported from *B. amyloliquefaciens* strains isolated from traditional soybean fermentation starter [31] and soil [32]. This apparently thermolabile protease of *B. amyloliquefaciens* UEF01 (Fig. 5) showed strong affinity with substrate casein (Fig. 6A & B). The inhibition of the enzyme by metal ions specifically by Mn and Na ions (Fig. 7) could not be explained during the present study. Inhibition of proteolytic activity of the cell-free culture filtrate of *B. amyloliquefaciens* UEF01 by PMSF, β-mercaptoethanol and EDTA, but not by 1,10-phenanthroline (Table 2) indicated its apparent similarity with the serine metalloprotease of *Halobacillus karajensis* [33]. However, to warrant such hypothesis conclusively a more detailed analysis seems to be essential.

5. CONCLUSION

Finally, it may be mentioned that this *in vitro* protease activity of the cell-free culture filtrate of the endophytic bacterium *B. amyloliquefaciens* UEF01 provides an indirect support on the prevalence of proteolytic enzymes in the host carnivorous plants as an essential requirement for prey digestion. However, this could only be confirmed through an in-depth plant-microbe interaction studies of the carnivorous plant *U. exoleta* R. Br. and their endophytic microbiota.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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