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Production of an extracellular neutral protease by *Bacillus aerius* UB02 endophytic to carnivorous plant *Utricularia stellaris*

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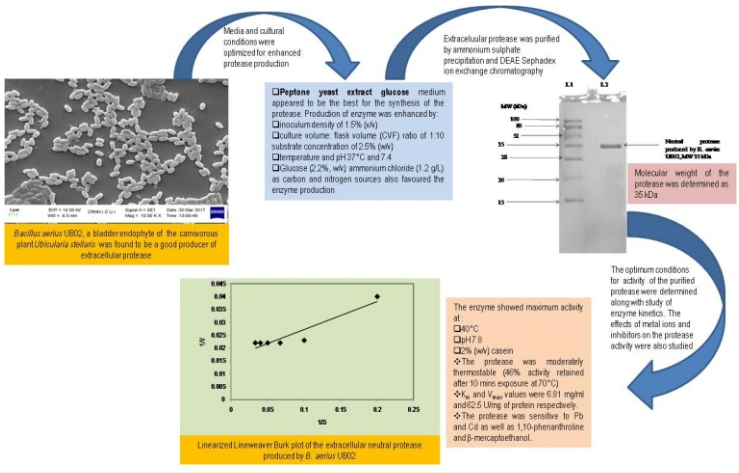
Abstract

Aim: Endophytic bacteria indigenous to carnivorous plants have been explored for production of novel bioactive metabolites including extracellular enzymes. *Bacillus aerius* UB02, an extracellular neutral protease producing isolate endophytic to bladder of *Utricularia stellaris* L. f. was used in this study.

Methodology: The bacterial isolate UB02 was identified following morphological, physiological, biochemical and 16S rRNA gene sequence analyses. The media as well as the cultural conditions for production of protease were optimized. The extracellular protease was isolated and purified from the cell-free culture filtrate by ammonium sulphate precipitation, dialysis and DEAE Sephadex ion exchange column chromatography and the optimum conditions for its activity were determined.

Results: The isolate *Bacillus aerius* UB02 (GenBank accession no. MK 696417, MCC accession no. 4132), produced significant amount of extracellular protease (38.29 U mg^{-1} protein) during growth in casein supplemented synthetic medium. However, peptone yeast extract glucose medium appeared to be the best for the synthesis of enzyme. Production of enzyme was enhanced by the inoculum density of 1.5% (v/v), culture volume: flask volume (CVF) ratio of 1:10, substrate concentration of 2.5% (w/v) with temperature and pH adjusted at 37°C and 7.4, respectively. Glucose (2.2%, w/v) and ammonium chloride (1.2 g/L) as carbon and nitrogen sources also favoured the enzyme production. The neutral protease with a molecular weight of approximately 35 kDa showed maximum activity at 40°C, pH 7.8 with 2% (w/v) casein. The enzyme exhibited K_m and V_{max} values of 6.81 mg ml^{-1} and 62.5 U mg^{-1} of protein, respectively, and was moderately thermostable. The protease activity was inhibited by Pb and Cd as well as 1,10-phenanthroline and β-mercaptoethanol.



Interpretation: These findings will help not only in understanding the role of endophytic bacteria and the enzymes produced by them in the digestion of prey by carnivorous plant but could also be explored for application in the field of biotechnology.

Key words: *Bacillus aerius,* Bacterial endophytes, Carnivorous plants, Enzyme kinetics, Extracellular protease, *Utricularia stellaris*

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Introduction

Exploitation of microbial enzymes in industrial sectors has widened the avenues for search of eco-friendly and sustainable bioprocesses (Banerjee and Ray, 2017; Razzaq *et al*., 2019). Interest of the scientific communities has been focussed towards production of novel enzymes with unique physiological properties and extreme environmental tolerance (Mienda and Yahya, 2011) to substantiate costly chemicals. Proteases are important hydrolytic enzymes utilized worldwide for breakdown of proteins and accounts for nearly 60% of the enzyme demand worldwide. Microbial proteases in particular play a pivotal role in food processing, processing of dairy products, preparation of pharmaceuticals, leather and textile industries (Gupta *et al*., 2002).

Proteolytic enzymes of microbial origin are essentially preferred over those of plant or animal proteases mainly due to rapid microbial growth rate, easy cultivation process, substrate specificity and effortless downstream processing for isolation and purification. Moreover, genetic manipulation of microorganisms often leads to alteration of enzymes desirable for industrial applications (Banerjee and Ray, 2017; Gupta *et al.,* 2002). The presence of functional groups and position of peptide bonds that determine microbial proteases are classified as acidic (pH 3.8 to 5.6), neutral (pH 5 to 8) and alkaline (pH 9 to 11) based on their optimal effectiveness in the reaction environment. Moreover, in microbes the intracellular proteases play a pivotal role in cellular differentiation, protein turnover and hormonal regulation, but the extracellular proteases find their application predominantly for protein hydrolysis (Adrio and Demain, 2014; Johnvesly and Naik, 2001; Razzaq *et al*., 2019).

Proteases, which are industrially important, are obtained from both bacterial as well as fungal sources, but majority of them are derived from strains belonging to the genus *Bacillus* (Pant *et al*., 2015; Elumalai *et al*., 2020). Screening of diverse groups of bacteria belonging to different environmental niches is a key factor towards isolating high yielding strains as well as identifying enzymes with unique properties. Several reports have established that the endophytic microbial community colonizing the internal tissues of plants produce a plethora of unique hydrolytic enzymes including proteases essential for their survival inside the plant environment (Zafaranloo *et al*., 2014; Mayerhofer *et al*., 2015; Ntabo *et al*., 2018; Castro *et al.*, 2014; Ek-Ramos *et al*., 2019; Chu *et al.,* 2019). Proteolytic activities have been recorded in bacteria associated with carnivorous species like *Sarracenia* (Scholtes and Kuserk, 2006) and *Nepenthes* (Li *et al*., 2012; Chan *et al*., 2016) that are likely to play beneficial role in digesting the prey and acquisition of nutrients for the host (Caravieri *et al.,* 2014). Likewise, while assessing the metabolic potentials of bacteria endophytic to *Drosera burmanii* and *Utricularia* spp., production of significant amount of proteases (Chaudhuri *et al.,* 2019) by 75% of the isolated strains has been already reported.

In this report, the isolation of a potent proteolytic endophytic bacterium *Bacillus aerius* UB02 from the bladder of *U. stellaris* L.f. has been studied and the cultural conditions for production of extracellular protease by this endophytic isolate have been optimized. Attempt has also been made to isolate and purify the extracellular protease elaborated by *B. aerius* UB02 and determine its characteristic features.

Materials and Methods

Bacterial culture and maintenance: The endophytic bacterium *Bacillus* UB02 obtained from the bladder of carnivorous plant *Utricularia stellaris* L.f. was used in this study. The endophytic isolate was maintained on slopes of nutrient agar by repeated sub-culturing.

Characterization and identification of the endophytic isolate: Morphological and physico-biochemical characteristics of *Bacillus* UB02 were determined following the standard microbiological methods (Gerhardt, 1994), while the antibiotic sensitivity was evaluated following the Kirby Bauer disc-diffusion method (Bauer *et al*., 1966). 16S rRNA gene sequence analysis was done by isolation of chromosomal DNA and purified according to the modified method of Murmur (1961).

PCR amplification was done using the universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'). Sequencing of the purified amplicon was carried out following Bi-directional DNA sequencing reaction using BDT v3.1 Cycle sequencing kit on ABI 3730 xl Genetic Analyzer. Consensus sequence of 16S rDNA was obtained and compared with closely related neighbour sequences retrieved from the NCBI database using BLAST search. Phylogenetic analysis was carried out using the software package MEGA6 after obtaining multiple alignments of the data available from the public databases by ClustalW (Thompson *et al*., 1994). Bootstrap analysis was made using 1000 replicates.

Kinetics of growth and protease production: Growth associated protease production by *Bacillus* UB02 was determined in mineral salts medium (containing g l^{-1}: K_2HPO_4, 0.6; KH_2PO_4, 3; $MgSO_4$, 0.1: NH_4Cl, 2; NaCl, 0.5; glucose, 0.8; pH 7) supplemented with 1% (w/v) casein. Erlenmeyer flasks were inoculated with overnight grown culture and incubated at 32°C under continuous shaking (120 rpm). Samples withdrawn at regular interval were assessed for growth and extracellular protease activity. Growth was measured by estimating the optical density at 540 nm and cell dry weight (CDW, g l^{-1}). Protease activity was assessed from cell-free culture filtrate following the method of Anson (1938).

Assay of protease activity: For determination of the protease activity, 200 µl of cell-free culture filtrate was added to 500 µl of

1% (w/v) casein in 50 mM phosphate buffer (pH 7), incubated at 40ºC for 20 min. The reaction was terminated by addition of 1 ml of 10% (w/v) trichloroacetic acid (Anson, 1938). The residual substrate was quantified following the methods of Lowry *et al*. (1951) and Folin and Ciocalteau (1927) using tyrosine as the standard. One unit (U) of protease is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute.

Total protein of cell-free culture filtrate was estimated according to Lowry *et al*. (1951) using bovine serum albumin (BSA) as standard. The total protease activity was divided by the amount of protein in the culture filtrate and expressed as specific activity of protease (U mg^{-1} of protein).

Optimization of conditions for production of protease: Cultural conditions involving growth media, inoculum concentration, aeration, temperature, pH, substrate concentration, and carbon and nitrogen sources for the production of extracellular protease by *Bacillus* UB02 were optimized under batch cultivation.

Isolation and purification of bacterial protease: The isolate UB02 was allowed to grow in Davis Mingioli's medium under optimized cultural condition. The extracellular protease was isolated from cell-free culture filtrate and partially purified following the method of Secades and Guijarro (1999). Ammonium sulphate was gradually added to the supernatant (at 4ºC) in a stepwise manner to achieve 30 - 90% saturation. The protein thus precipitated is separated by centrifugation (10,000 rpm, 10 min, 4ºC), pooled, dialysed in double distilled water at 4ºC and dissolved in phosphate buffer (pH 7) for quatification of protease enzyme activity.

The dialysate was loaded to DEAE Sephadex column pre-equilibriated with phosphate buffer (pH 7) and the enzyme was eluted with the same buffer at a flow rate of 1 ml min^{-1}. The purified protease was lyophilized at -56ºC (LSL Secfroid, SICO) and stored at -20 ºC until further use.

Determination of properties of the purified bacterial protease: Molecular weight of the purified protease was determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Sample to be loaded in the well of SDS matrix was prepared by mixing purified protease with 10 mM Tris HCl (pH 8), 2.5% SDS, 5% β-mercaptoethanol and 0.002% bromophenol blue. The gel was stained with Coomassie brilliant blue and protein bands were observed under transilluminator.

Effect of several factors such as temperature, pH, substrate concentration, inhibitors and metal ions were studied with the partially purified protease.

Statistical analysis: All the experiments were performed in triplicates and the data shown represent the mean value ± standard deviation (Altman and Bland, 2005).

Results and Discussion

The Gram-positive endospore forming motile rod-shaped endophytic bacterium isolated from the bladder of *U. stellaris* produced many enzymes like catalase, lipase, caseinase and gelatinase. It was able to ferment only glucose but could utilize galactose, sucrose, mannitol, sorbitol, fructose, arabinose, mannose and trehalose. The isolate UB02 was sensitive to penicillin G, ampicillin, ciprofloxacin, rifampicin, gentamycin, trimethoprim, vancomycin, tetracycline, kanamycin, and novobiocin. It was resistant to erythromycin and chlortetracycline (Table 1).

The 16S rDNA sequence (1579 bp) of *Bacillus* UB02 showed 100% sequence similarity with that of *Bacillus aerius* available in the NCBI databases and was designated as *Bacillus aerius* UB02. The nucleotide sequence of 16S rRNA gene and the viable culture of *Bacillus aerius* UB02 were submitted to the GenBank database and to Microbial Culture Collection (MCC) Pune, India with the Accession numbers MK 696417 and MCC 4132 respectively.

The ability to produce hydrolytic enzymes, particularly protease, by bacteria endophytic to plants growing under diverse ecological conditions has been well documented (Carrim *et al.,* 2006; Castro *et al*., 2014). Contrary to these, reports on the presence of proteolytic bacteria present in carnivorous plants, their involvement in protease secretion and digestion of prey are

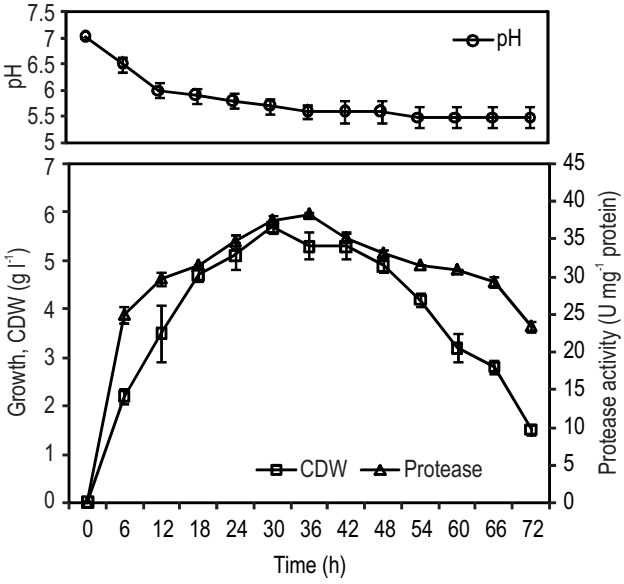

Fig. 1 : Time course of growth and protease production by *B. aerius* UB02 in casein supplemented mineral salts medium; 1 U = amount of enzyme that releases 1 µg of tyrosine per ml per min

Table 1 : Morphological, physio-biochemical characters and antibiotic sensitivity profile of endophytic bacterial isolate *Bacillus* Ub02

Character	Response	Character	Response	
Colony morphology	Smooth, white	Fermentation and utilization of	Fermentation	Utilization
Cell morphology	Single Rods, 2.5-3.7 X 1.2-1.8 µm	Glucose	+	+
Gram nature	Gram +ve	Maltose	-	-
Motility	+	Sucrose	-	+
Endospore formation	+, central	Mannitol	-	+
Production of		Sorbitol	-	+
catalase	+	Fructose	-	+
caseinase	+	Galactose	-	+
oxidase	-	Lactose	-	-
amylase	-	Rhamnose	-	-
gelatinase	+	Raffinose	-	-
pectinase	-	Arabinose	-	+
urease	-	Aldonitol	-	-
nitrate reductase	-	Dulcitol	-	-
cellulase	-	Trehalose	-	+
lipase	+	Inositol	-	-
Indole production	+	Mannose	-	+
Citrate utilization	+	Resistant to antibiotics	E, Ct	
Range of temp. for growth	32-42° C	Sensitive to antibiotics	Van, Tet, K, Nov, P, Amp, Cip, Rif, Gen, Tri	
Range of pH for growth	7-9			
NaCl tolerance	5% (w/v)			

Antibiotics: E= erythromycin, P= penicillin G, Amp= ampicillin, Cip= ciprofloxacin, Rif= rifampicin, Gen= gentamycin, Tri= trimethoprim, Van= vancomycin, Tet= tetracycline, K= kanamycin, Ct= chlortetracycline, Nov= novobiocin; + = positive and – = negative response

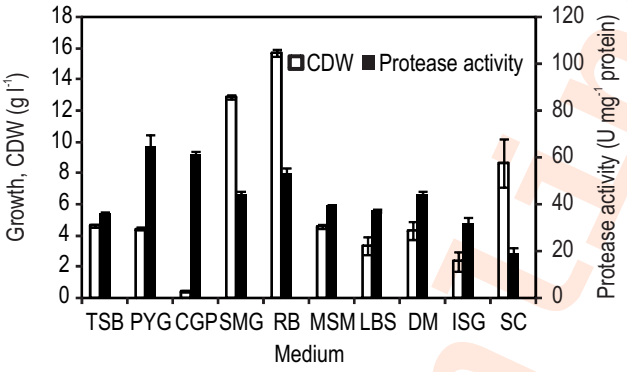

Fig. 2 : Effect of different culture media on growth and production of protease by B. aerius UB02; 1 U = amount of enzyme that releases 1µg of tyrosine per ml per min; Media used: TSB - tryptic soy broth, PYG - peptone yeast extract glucose, CGP - casein hydrolysate glucose peptone, SMG - soyabean meal glucose, RB - rice bran urea medium, MSM - mineral salts medium, LBS - Lindenbein synthetic medium, DM - Davis Mingiol's medium, ISG -inorganic salt glucose medium, SC - starch calcium carbonate medium

scanty (Takeuchi *et al*., 2011; Lee *et al*., 2014; Chaudhuri *et al*., 2017). This communication, as far as we are aware reports production and partial characterization of an extracellular protease by an endophytic *Bacillus* strain UB02 isolated from the bladders of *U. stellaris* (Chaudhuri *et al*., 2019) for the first time.

Members of the genus *Bacillus,* the most predominant amongst the endophytic community have been recognized as versatile metabolite producers (Reinhold-Hurek and Hurek, 2011; Frank *et al*., 2017; Ek-Ramos *et al.,* 2019) which includes hydrolytic enzymes, especially proteases. The endogenous occurrence of *Bacillus aerius* has been reported by few authors in crop plants as well as halotolerant species where they play significant role as plant growth promoting endophytes as well as improvement of salt tolerance in plants (Arora *et al*., 2014). However, reports showing caseinolytic protease production by *B. aerius* is rare (Ananthanarayan and Dubhashi, 2015).

Time course of growth-associated production of extracellular protease by *B. aerius* UB02 in mineral salts medium containing 1% casein revealed accumulation of both biomass and protease (total activity 31.4 U ml^{-1} and specific activity 38.29 U mg^{-1} protein) at 36 hr of incubation which occurred at the end of log phase (Fig. 1). Following this, a decline was observed in both growth and protease production.

To determine a suitable medium for the maximum growth and production of protease, *B. aerius* UB02 was grown in different synthetic and complex media, each supplemented with 1% casein. Rice bran urea medium followed by soybean meal glucose medium were observed to be best suited for growth (CDW, 15.74 g l^{-1} and 12.88 g l^{-1}), while peptone yeast extract

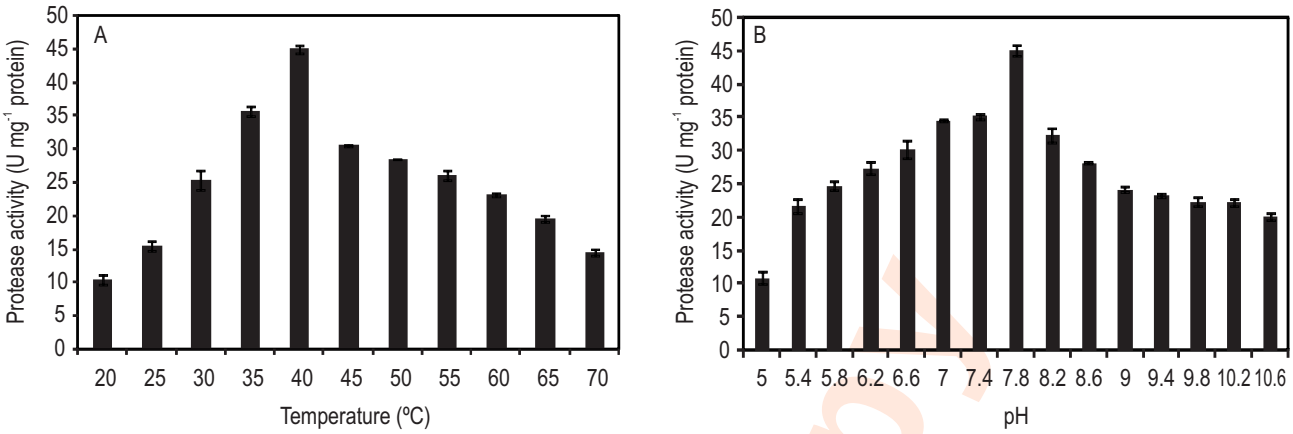

Fig. 3 : Effect of temperature (A) and pH (B) on the activity of purified protease of *B. aerius* UB02; 1 U = amount of enzyme that releases 1µg of tyrosine per ml per min.

Table 2 : Optimization of cultural conditions for growth and production of protease by *B. aerius* UB02

Parameters	Test range	Optimized parameter	Growth, CDW (g l^{-1})	Protease (U mg^{-1} protein)
Inoculum (%, v/v)	0.5 - 2	1.5	4.6±0.14	46.21±0.23
Aeration (CVF)	1:10, 1:5, 2:5, 3:5, 4:5	1:10	4.7±0.28	47.52±0.9
Temperature (°C)	28 - 42	37	4.6±0.28	47.49±1.18
Casein (%, w/v)	0.5-3	2.5	4.7±0.28	49.38±1.16
pH	5 - 10.6	7.4	5.4±0.20	48.19±0.59
Carbon source	glu, gal, suc, ara, fru, tre, mannl, man, sor	Glucose	4.6±0.28	44.84±1.02
Glucose (%, w/v)	1.6 - 2.4	2.2	4.85±0.06	49.57±0.35
Nitrogen source	Peptone, tryptone, casamino acid, yeast extract, NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, NH_4NO_3	NH_4Cl	4.6±0.28	49.02±0.56
NH_4Cl (g l^{-1})	0.6 - 1.4	1.2	5.7±0.06	48.25±0.33

Values represent mean of triplicate experiments ± standard deviation; 1 U = amount of enzyme that releases 1µg of tyrosine per ml per min; 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

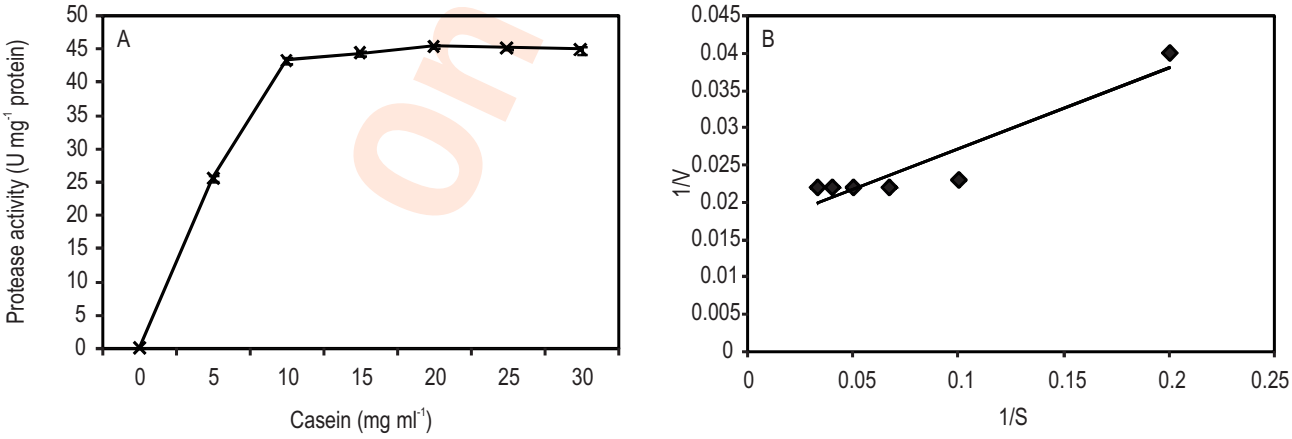

Fig. 4 : Effect of substrate concentration on the activity of protease produced by *B. aerius* UB02 (A) and linearized Lineweaver-Burk plot for enzyme activity (B); 1 U = amount of enzyme that releases 1µg of tyrosine per ml per min.

Table 3 : Effect of different inhibitors on activity of the protease produced by *B. aerius* UB02

Inhibitor	Concentrations (mM)	Protease activity (U mg^{-1} protein)	% inhibition
Control	-	44.11	0
Ethylene diamine tetraacetic acid	1	21.90	50.35
	2	18.39	58.26
	5	17.48	60.32
	10	15.73	64.29
β-mercaptoethanol	1	26.26	40.39
	2	25.21	42.77
	5	23.92	45.70
	10	21.06	52.39
Phenyl methyl sulfonyl fluoride	1	24.70	43.93
	2	22.36	49.24
	5	15.08	65.77
	10	10.72	75.67
1,10-phenanthroline	1	16.90	61.64
	2	14.82	66.36
	5	10.78	75.53
	10	6.69	84.81

1U = amount of enzyme that releases 1 µg of tyrosine per ml per min

glucose and casein hydrolysate glucose peptone media were most favourable for production of protease by UB02 (specific activity of 65 and 44.13 U mg^{-1} protein, respectively) (Fig. 2).

Several other physico-chemical parameters influencing growth and protease production by *B. aerius* UB02 were also evaluated and the optimized conditions are summarized in Table 2. Both growth and production of extracellular protease was maximum at 1.5% inoculum concentration (46.21 U mg^{-1} protein), culture volume by flask volume (CVF) ratio of 1:10 (47.52 U mg^{-1} protein), a temperature of 37ºC (47.49 U mg^{-1} protein) and pH of 7.4 (48.19 U mg^{-1} protein). Though growth of the isolate did not vary much above 1.5% casein, the highest protease activity (49.38 U mg^{-1} protein) was recorded at 2.5% casein. Glucose (2.2%) and ammonium chloride (1.2 g l^{-1}) appeared to be the best utilized carbon and nitrogen sources. The above studies revealed the dependence of *B. aerius* UB02 on nutritionally complex medium along with glucose and ammonium chloride as carbon and nitrogen sources, respectively. Moreover, the pH and temperature requirement indicated the mesophilic and neutrophilic nature of the producer organism.

The isolate *B. aerius* UB02 was cultured in Davis Mingioli's medium under optimized cultural conditions for 36 hr and the extracellular protease was isolated from 500 ml of cell-free culture filtrate following ammonium sulphate precipitation (30-90% saturation). The maximum specific activity (44.11 U mg^{-1}) of the protease was found at 70% saturation level as compared to 38.29 U mg^{-1} of protein from the crude culture filtrate. The precipitates were pooled, dialysed and lyophilized as per the methods described. The partially purified enzyme was dissolved in 50 ml of phosphate buffer and purified using DEAE Sephadex column chromatography.

The molecular weight of purified protease produced by *B. aerius* UB02 as detected using SDS-PAGE was approximately 35 kDa. Extracellular protease production has been reported from several species of *Bacillus* which have been categorized into two major types, alkaline protease and neutral protease, that have been reported to possess importance in food and detergent industries (Rani *et al.,* 2012; Razzaq *et al.*, 2019; Thomas *et al.,* 2021). A thermostable neutral protease reported from *B. stearothermophilus* showed a molecular mass of 38 kDa while that from B. subtilis showed a molecular weight of 65.4 kDa in SDS-PAGE (Razzaq *et al.*, 2019).

On evaluating the effect of temperature on protease activity in the range of 20°-70°C, activity was found to increase with increase in temperature and was maximum at 40°C (45.05 U mg^{-1} protein). It was followed by a decline thereafter (Fig. 3A). Likewise, when the activity was tested in the pH range of 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 8-10.6) buffers, the highest activity (44.96 U/mg of protein) was evident at pH 7.8 (Fig. 3B). The protease so isolated from *B. aerius* UB02 in this present study appears to be a neutral protease. Reports suggest that these neutral proteases could be valuable in food industry, particularly due to the fact that they generate less bitterness in hydrolysing the proteinaceous componenets in food and have medium rate of

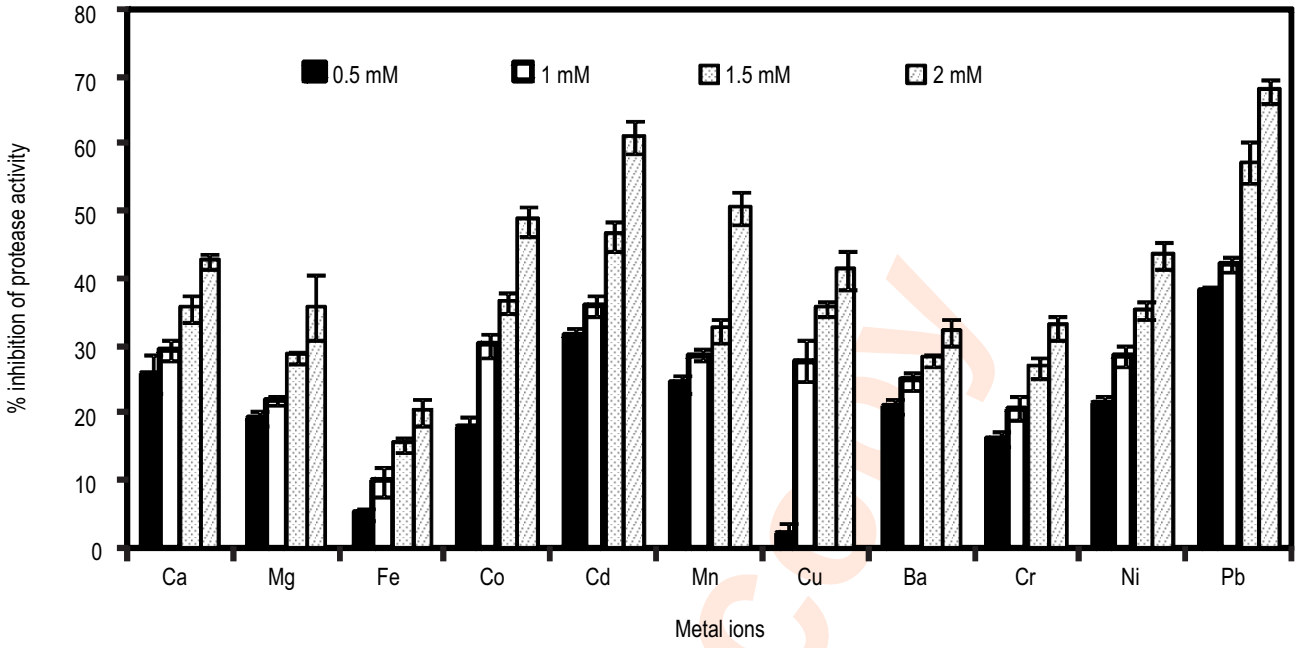

Fig. 5 : Effect of metal ions on the activity of protease produced by *B. aerius* UB02

reactions. They are also used in the brewing industry as they are insensitive to plant proteinase inhibitors (Razzaq *et al*., 2019).

Thermostability of protease in UB02 was studied by pretreatment at 40°C-70°C for 5-15 min and subsequently tested for activity following the usual procedure. The protease activity declined with increase in temperature as well as duration of pretreatment. However, the enzyme retained nearly 52% and 46% of its activity when treated at 60°C and 70°C for 10 min, respectively.

The specific activity of *B. aerius* UB02 protease increased with increasing concentrations of casein and the maximum activity (45.44U mg^{-1} of protein) was observed at 2% (w/v) casein showing a strong affinity towards the substrate (Fig. 4A). The enzyme kinetics followed a linearized Lineweaver-Burk plot (Fig. 4B) and the K_m and V_{max} of protease was 6.81 mg ml^{-1} and 62.5 U mg^{-1} protein, respectively. The protease bears striking resemblance to neutral proteases produced by *B. subtilis* (Pant *et al*., 2015) and *B. amyloliquefaciens* (Wang *et al*., 2016).

Presence of different metal ions like Ca, Mg, Fe, Co, Cd, Mn, Cu, Ba, Cr, Ni, Pb (0.5-2 mM) as chloride salts inhibited the protease activity (Fig. 5). Pb was most inhibitory (68.25%) at the highest concentration (2mM), while protease was least affected (20.27%) by Fe. The effect of metals is in the following order: Pb>Cd>Mn>Co>Ni>Ca>Cu>Mg>Cr>Ba>Fe according to their degree of inhibition. The inhibition by heavy metal ions in general may be due to enzyme inactivation following their strong binding with sulphydryl groups (Mizrahi and Achituv, 1989).

Presence of EDTA, β-mercaptoethanol, PMSF and 1, 10-phenanthroline in the reaction mixture inhibited the activity of protease of *B. aerius* UB02 (Table 3). The inhibition was maximum (84.81%) at 10 mM 1,10-phenanthroline, and was moderate in the presence of β-mercaptoethanol. Inhibition of proteolytic activity by 1,10-phenanthroline (Table 3) also indicated its apparent similarity with the neutral protease secreted by *B. subtilis* (Feder *et al*., 1971).

The production and characterization of a neutral protease by endophytic *Bacillus aerius* UB02 having low thermotolerance property appears to be significant particularly in food industry for hydrolysis of food proteins and in brewing. The present study on the neutral protease produced by the bacterium *B. aerius* UB02 inherent of the bladder of *U. stellaris*, provides an indirect evidence in favour of the involvement of endogenous endophytic bacterial strains in prey digestion and host plants nutrition. However, detailed studies are warranted to justify and establish such involvement.

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Add-on Information

Authors' contribution: M. Chaudhuri: Performed experiments, wrote MS, **A.K. Paul:** Designed experiments, checked the MS, **A. Pal:** Analysed the results, checked MS, statistical analysis.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not applicable

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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