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Research Article

Screening and Enumeration of Rhizospheric Soil Bacteria for Production of Extracellular Protease

Manaswita Mukherjee and Arundhati Pal*

Article Info

Post Graduate Department of Botany, Serampore College, 9 William Carey Road, Serampore, Hooghly, West Bengal - 712201, INDIA

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*Correspondence: **Arundhati Pal**

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Abstract

Plant rhizosphere is an important ecological niche having a rich array of plant released metabolites that provide carbon and nutrients for augmentation of a bioactive microbial zone. This diverse range of bacteria, including protease producers, plays a central role in nutrient cycling and biodegradation of organic matter. A comprehensive study was carried out to enumerate cultivable microflora underneath *Areca catechu* L., *Averrhoa carambola* L., *Mangifera indica* L. and *Cycas circinalis* L. and primarily screen their protease-producing ability in casein supplemented nutrient agar. It was observed that 85.2 % of 27 bacterial strains produced extracellular protease out of which six strains showed good proteolytic index (>1.7). Bacterium *Bacillus* CGR04 from mango rhizosphere, showed highest protease production under submerged growth in mineral salts medium. Optimal enzyme production occurred during the early stationary phase (24 h) in presence of 1% casein. *Bacillus* CGR04 showed maximum proteolytic activity at 1.2% (w/v) substrate (335.86 U/ml) and enzyme kinetics followed a linearized Line weaver Burk plot. The Km and Vmax values were calculated as 4.77 mg/ml and 454.55 U/ml, respectively. Enzyme production was optimal in presence of 1 % (w/v) glucose and peptone showing protease production of 279.6 U / ml and 304.84 U/ml respectively. Moreover, the study revealed that enzyme production was optimum at pH 7.5 (307.1 U / ml) and 35ºC (297.3 U / ml) indicating production of a neutral protease. Furthermore, purification and characterization of the protease from *Bacillus* CGR04 could be of potential application for dairy and beverage industries.

Keywords: Rhizobacteria; Protease production; Caseinase; *Bacillus*; Proteolytic index; Optimization

Running Title: Neutral protease production by rhizobacteria

Introduction

Protease, also known as peptidase or proteinase, are known to degrade complex proteins into polypeptide molecules or forming monomeric units of amino acids. Various forms of proteolytic reactions are noted, however, the enzyme can be broadly classified as either endopeptidase or exopeptidase. They are ubiquitously produced by plants (bromelain, keratinase and ficin), animal (trypsin, chymotrypsin, pepsin, etc) as well as microorganisms of which industrially useful proteases are obtained usually from bacterial or fungal fermentations. Worldwide demand of proteases of different nature remains insufficient and hence scientists and researchers have focused their attention towards isolation of novel protease producers [1, 2].

Microbial enzymes are regarded as "green alternatives" to the existing chemicals for catalyzing chemical reactions for production of economically important products having high commercial values. Extracellular production of proteases having high biological activity depends on isolation and identification of a potential strain which is able to produce the enzyme under solid-state or submerged fermentation systems. Microbial proteases have been broadly classified into families based on their structure, amino acid sequences or behavior at different pH range. Alkaline protease producing bacteria and fungi belonging mainly to the genera *Bacillus* and *Aspergillus* have been not only being isolated from soil, water, lakes, etc.

but were also reported to be entomopathogenic or endophytic / symbiotic ones. Neutral proteases have been found to be active at weak acidic or alkaline pH and are mostly suitable for use in food industries [3, 4].

The industrial use of protease has been found mostly for production of food and beverages, dairy, pharmaceuticals, leather tanning, silk degumming, textile units, biofuel, animal feed and home cleaning. The main advantage behind using microbially derived enzyme remained low production cost and high yields. With the advent of a more widespread ability to adapt better stability, specificity and selectivity of the enzyme, the commercial future of microbial-derived proteases appears to be a hopeful one [5, 6].

Several protease-producing bacteria and fungi have been isolated from different environmental niches and the optimal conditions for enzyme production and bioactivity has been studied. Several pathogenic bacteria have also been found to be good producer of the enzyme; however, they cannot compete as industrial strains. Researchers are continuously looking for novel protease producing strains that tolerate hash environmental conditions, prevent autoproteolytic cleavage, large substrate specificity as well as stability under optimum pH and temperature. Protease producing bacteria represented 18 – 58 % of the total microbial population isolated from rhizoplane of different crop plants. The surface of the plant roots and root exudates along with growth conditions of the crop influences the bacterial communities for enzyme production [7].

Genotypic and phenotypic profiles of protease producing bacteria from rhizosphere of apple, banana, rice, wheat, groundnut, mangrove, sugar beet and cotton have been reported from different parts of the world including India. Majority of the isolates were found to produce alkaline protease and belonged to the genera *Bacillus* [4, 8].

However, neutral protease producers are also not uncommon and many also showed antagonistic activity against phytopathogenic organisms [9]. The present study aims to enumerate the diversity of culturable proteolytic bacteria from rhizospheric soil of commonly occurring garden plants and optimize the cultural conditions for extracellular protease production during growth.

Materials and Methods

3.1. Collection of soil samples and isolation of bacteria:

Soil samples were collected in spring from the rhizosphere of *Areca catechu* (Arecaceae), *Averrhoa carambola* (Oxalidaceae), *Mangifera indica* (Anacardiaceae) and *Cycas circinalis* (Cycadaceae) growing in the garden of Serampore College campus and Chandannagar, West Bengal, India. Soil was collected from a depth of 10 cm in sterile glass containers and immediately brought to the laboratory for microbiological analysis. Total culturable bacteria were analyzed following serial dilution and plate count technique in nutrient agar medium. The antibiotic gresiofulvin (50 µg/ml) was used in the sterile medium to inhibit growth of fungal members. The plates were incubated at 30ºC for 48 -96 h and observed for growth of bacterial isolates. Bacterial colonies differing in morphological characteristics were isolated, pure cultures were developed by dilution and streaking method on isolation medium and repeatedly sub-cultured at 4 weeks interval.

3.2. Characterization of rhizospheric bacteria:

The bacterial isolates obtained in pure form were characterized micromorphologically and physio-biochemically following standard microbiological methods like Gram staining, endospore staining, production of biochemical enzymes, fermentation of sugars, nitrate reduction, utilization of citrate, ornithin, production of urease, etc. as well as antibiotic sensitivity test following Kirby Baur disc diffusion assay using antibiotic impregnated discs (Himedia, 6 mm). Antibiotics used included Ampicillin (25µg), Bacitracin (10µg), Chloramphenicol (30µg), Chlortetracycline (30µg), Ciprofloxacin (30µg), Erythromycin (25µg), Kanamycin (30µg), Novobiocin (30µg), Rifampicin (30µg), Streptomycin (25µg), Tetracycline (30µg) and Vancomycin (30µg).

3.3. Primary screening of bacteria for their proteolytic activity:

Rhizospheric bacteria were primarily screened for their ability to produce extracellular protease in solid state fermentation using casein as substrate. The isolates were spot inoculated on nutrient agar medium supplemented with 1% (w/v) casein and incubated for 48 h at 30°C. The plates were flooded with 10% HCl (v/v) and observed for the formation of clear zone surrounding the bacterial growth. Proteolytic index was calculated as the ratio of the diameter of clear zone including bacterial growth to that of the diameter of bacterial growth.

3.4. Protease production in submerged fermentation:

Extracellular protease production was assayed in mineral salts medium containing (g/L) peptone 5.0; $MgSO_4.7H_2O$ 5.0; K_2HPO_4 2.0; $CaCl_2$ 1.0; glucose 10 and soluble casein 10 (pH 7.0). Erlenmeyer flasks (100 ml) containing 20 ml mineral salts broth was inoculated with freshly grown inoculum (1 % v/v) and incubated at 30ºC on a rotary shaker. Aliquot (5 ml) was withdrawn aseptically and growth was measured by estimating the optical density of the medium at 540 nm using Systronics colorimeter (CL223). The culture was centrifuged (10,000 rpm, 10 min) using Remi RM12C centrifuge and extracellular protease enzyme was assayed following the modified method of Anson [10] using tyrosine as standard.

3.5. Assay for protease

Protease activity was assayed using cell-free culture filtrate following the modified method of Anson (10). About 500 µl of 1% (w/v) casein in 50 mM phosphate buffer (pH 7.0) was taken and 500 µl of the cell-free culture filtrate was added. The reaction mixture was incubated at 45°C for 60 min in a water bath. The reaction was terminated by adding 500 µl 10% (w/v) trichloroacetic acid and kept at room temperature for 15 min. The unreacted substrate was separated by centrifugation at 10,000 rpm for 10

min. The residual substrate was quantified following method of Lowry *et al*. [11] using tyrosine as the standard. One unit (U) of protease was defined as the amount of enzyme that releases 1 µM of tyrosine per ml per h.

3.6. Optimization of cultural conditions for protease production:

The optimal parameters for extracellular protease production were performed following OVAT (One variable at a time) method. The variables included incubation time; substrate concentration, carbon and nitrogen concentration, pH of the medium and temperature for growth. Enzyme kinetics was studied by generating Lineweaver Burk plot to estimate the velocity maxima and Michelis Menten constant.

3.7. Statistical analysis:

All experiments were conducted in triplicates and results represent mean ± standard deviation.

Results and Discussion

4.1 Microbiological analysis of rhizospheric soil:

A total of four rhizospheric soil samples were collected from underneath *Areca catechu* L. (betel palm), *Averrhoa carambola*L. (star fruit), *Mangifera indica* L. (mango) and *Cycas circinalis* L. (sago palm) were subjected to microbiological analysis (Table 1). The microbial load of mesophilic heterotrophic bacteria was found to be highest underneath *Averrhoa carambola* (16.5×10^6 CFU per g soil) followed by *Areca catechu* (5.4×10^6); *Cycas circinalis* (4.7×10^6) and *Mangifera indica* (4.15×10^6). A total 27 phenotypically distinguishable bacterial isolates were obtained in pure form showing that rhizosphere is a dynamic ecological niche where several plant derived molecules function between the roots of higher plants and root associated microorganisms that contribute to effective plant protection and mitigation of plant nutrients [12]. Several reports have identified indigenous and novel bacteria including actinobacteria from rhizosphere of star fruit and betel palm [13, 14]. However, in the present study no attempt was made to use selective media for isolation of filamentous actinobacteria.

Table 1: Microbiological analysis of rhizospheric soil samples

Plant (family)	Location	Total Count, CFU / g soil	No. of bacterial cultures
***Areca catechu* L. (Arecaceae)**	Chandannagar (22.8671° N, 88.3674° E)	5.4×10^6	05
***Averrhoa carambola* L. (Oxalidaceae)**	Serampore (22.7519° N, 88.3518° E)	16.5×10^6	05
***Mangifera indica* L. (Anacardiaceae)**	Chandannagar (22.8671° N, 88.3674° E)	4.15×10^6	06
***Cycas circinalis* L. (Cycadaceae)**	Serampore (22.7519° N, 88.3518° E)	4.7×10^6	11
Total number of bacterial cultures obtained in pure form			**27**

CFU = colony forming unit; Total count was estimated following serial dilution and direct plate count technique on nutrient agar medium. The plates were incubated in 30°C for 2-4 days.

4.2 Characterization of rhizospheric bacteria:

Twenty-seven rhizospheric bacterial isolates were subjected to micro-morphological characterization following standard protocol (Table 2).

Table 2: Micro-morphological characterization of the mesophilic heterotrophic bacterial isolates isolated from rhizospheric soil

Sl. No.	Plant	Isolate	Colony morphology	Diffusible pigment	Cell morphology		Gram nature	Endospore formation
					Cell shape	Size (lxb), µm		
1	*Mangifera indica* L.	CGR01	Cream, smooth, entire, raised	-	Bacilli, in chains	2.8-5.6 x 1.4	+	+
		CGR02	Yellowish, rough, irregular, flat	-	Short rods, 2-4 cells in chain	1.4-2.6 x 1.4	-	-
		CGR03	White, rough, entire, flat	-	Rods in chains	4.2-7 x 1.4-2.8	+	+
		CGR04	Cream, wrinkled, undulate, irregular margin, umbonate	-	Rods in chain of 2-6 cells, rarely solitary	2.8-4.2 x 0.7-1.4	+	+
		CGR06	Cream, rough, filamentous, flat	-	Rods, solitary or in chains	1.4-4.2 x 0.7-1.4	+	+
		CGR07	Cream, smooth, regular, flat	-	Rods mostly in chains	2.8-5.6 x 1.4-2.8	+	+
2	*Averrhoa carambola* L.	SCA01	Yellow, smooth, curled, flat	-	Short rods mostly in groups	1.4-2.8 x 0.7-1.4	+	-
		SCA02	White, margin filamentous, raised	-	Short rods, in chains of 2-4 cells	2.8-4.2 x 1.4	+	+
		SCA03	Cream, smooth, lobate, flat	-	Rods mostly solitary, or groups	1.4-2.8 x 0.7-1.4	+	+
		SCA04	Cream, smooth, regular, flat	-	Rods mostly in chains	2.8-5.6 x 1.4	+	+
		SCA05	Yellowish white, smooth, undulate, flat	-	Rods mostly in pairs, rarely solitary	4.2-7 x 0.7-1.4	+	+
3	*Areca catechu* L.	SCB01	White, wrinkled, undulate, flat	-	Rods in chains of 4-6 cells	2.8-5.6 x 1.4	+	+
		SCB02	Cream, smooth, entire, flat	-	Cocci mostly in chains	2.4-4.2 x 0.7	+	-
		SCB03	White, smooth, lobate, flat	-	Rods, in chains of 4-8 cells	1.4-5.6 x 1.4	+	+
		SCB04	White, wrinkled, entire, flat	-	Rods mostly in pairs	2.8-5.6 x 1.4	+	+
		SCB05	Cream, rough, entire, flat	-	Coccobacilli, in chains of 5-7 cells	1.4-1.8 x 0.7-1.4	-	-
4	*Cycas circinalis L.*	SCC01	Creamish yellow, rough, undulate	+	Rods mostly in chains	7.6 x 1.52	+	+
		SCC02	Creamish yellow, undulate, flat	+	Rods in chains of 4-6 cells	6.08 x 1.52	+	-
		SCC03	Yellowish, smooth, undulate, flat	+	Rods in chains of 2-4 cells	4.56 x 1.52	+	-
		SCC04	Cream, smooth, undulate, flat	+	Rods mostly in chains	4.56 x 1.52	+	+
		SCC05	White, rough, filamentous, raised	+	Rods mostly in pairs, or solitary	4.56 x 1.52	-	-
		SCC06	Cream, smooth, undulate, flat	+	Rods mostly in pairs,	4.56 x 1.52	+	+
		SCD01	Cream, rough, lobate, flat	-	Coccobacilli, mostly in pairs	2.8 x 2.4	-	-
		SCD02	Yellow, rough, undulate, raised	+	Rods in chains of 4-6 cells	3.95 x 1.52	+	+
		SCD03	Cream, wrinkled, undulate	+	Rods in chains of 4-6 cells	6.84 x 1.52	+	+
		SCD04	Cream, rough, undulate, raised	**+**	Short rods, in pairs, or solitary	3.04-5.6 x 1.4-2.8	**-**	**-**
		SCD05	Yellowish, smooth, undulated, flat	**+**	Short rods, in pairs	3.8-5.6 x 1.4-2.8	-	-

Majority of the mesophilic heterotrophic bacteria were found to be Gram-positive rod shaped (>80 %) cells and seventeen of them were tentatively identified as *Bacillus* due to their endospore formation. Ten bacteria showed production of diffusible pigments. Several reports also demonstrated occurrence of Gram-positive bacteria including actinobacteria from rhizospheric soil of betel palm [13], mango [15] and star fruit [14] trees.

4.3 Screening of rhizospheric bacteria for extracellular protease production:

All twenty-seven rhizospheric bacterial isolates were subjected to primary screening for production of extracellular protease enzyme following spot inoculation on nutrient casein agar plate. It was observed that 85.18% of 27 isolates exhibited protease production by showing formation of clear zone surrounding the bacterial growth and proteolytic index was found to range from 1.25 to 2.05 (Table 3). Several reports have shown rhizobacterial strains producing different hydrolytic enzymes as well plant growth promoting traits that are applicable for numerous industrial applications in food, pharmaceuticals sustainable agriculture worldwide [4]. But this study shows the first report on protease production from bacterial colonizing underneath mango, areca nut or star fruit trees.

4.4 Categorization of rhizobacterial isolates:

Based on the proteolytic index (PI), the rhizobacterial isolates were categorized into poor (PI < 1.4), moderate (PI 1.4 -1.7) and good (PI > 1.7) protease producers. Six strains (two each from mango and star fruit tree and one each from areca nut and *Cycas*) representing 22.3 % of total isolates showed good proteolytic index (1.71 – 2.05) (Table 4). These potent protease producers were further subjected to submerged fermentation for growth associated protease production.

Table 3: Screening of rhizobacterial isolates for extracellular proteolytic activity

Plant	Isolate no.	Diameter of Bacterial growth, mm	Diameter of clear zone including bacterial growth, mm	Protease Response	Proteolytic index
***Mangifera indica* L.**	CGR01	9.0 ± 1	16.0 ± 0.0	++	1.80 ± 0.2
	CGR02	16.5 ± 2.5	21.0 ± 3.0	+	1.27 ± 0.01
	CGR03	9.5 ± 1.5	-	-	-
	CGR04	11.5 ± 0.5	21.5 ± 0.5	++	1.86 ± 0.03
	CGR06	7.5 ± 1.5	-	-	-
	CGR07	6.0 ± 0.1	-	-	-
***Averrhoa carambola* L.**	SCA01	13.0 ± 0.1	16.5 ± 5.5	++	1.68 ± 0.11
	SCA02	12.0 ± 1.0	17.5 ± 2.5	+	1.44 ± 0.08
	SCA03	12.0 ± 0.2	21.5 ± 3.5	++	1.79 ± 0.01
	SCA04	14.0 ± 0.4	23.5 ± 5.5	++	1.71 ± 0.09
	SCA05	12.5 ± 0.5	16.0 ± 1.0	+	1.28 ± 0.13
***Areca catechu* L.**	SCB01	11.0 ± 0.0	19.0 ± 1.0	++	1.72 ± 0.09
	SCB02	12.0 ± 0.1	17.5 ± 1.5	+	1.45 ± 0.01
	SCB03	10.0 ± 0.2	15.0 ± 0.0	++	1.56 ± 0.31
	SCB04	12.0 ± 0.2	17.5 ± 1.5	+	1.47 ± 0.12
	SCB05	9.5 ± 0.5	15.5 ± 0.5	++	1.63 ± 0.13
Cycas circinalis L.	SCC01	14.0 ± 0.1	18.0 ± 1.0	+	1.29 ± 0.16
	SCC02	9.0 ± 0.0	14.5 ± 1.5	++	1.60 ± 0.16
	SCC03	9.0 ± 0.0	18.5 ± 0.5	++	2.05 ± 0.05

	SCC04	10.5 ± 0.5	15.0 ± 0.0	+	1.43 ± 0.07
	SCC05	10.5 ± 1.5	13.5 ± 1.5	+	1.29 ± 0.04
	SCC06	10.0 ± 0.0	12.5 ± 0.5	+	1.25 ± 0.05
	SCD01	8.5 ± 1.5	-	-	-
	SCD02	13.0 ± 0.2	21.0 ± 4.0	++	1.60 ± 0.06
	SCD03	11.0 ± 0.1	15.5 ± 0.5	+	1.41 ± 0.08
	SCD04	10.0 ± 0.1	15.0 ± 1.0	++	1.50 ± 0.05
	SCD05	10.5 ± 0.5	14.5 ± 0.5	+	1.38 ± 0.02

"-"= No response; "+"= weak to moderate response; "++"= good positive response

Proteolytic assay was conducted in casein supplemented nutrient agar plates. The plates were incubated in 30^{o}C for 48 h and clear zone was detected using 10% hydrochloric acid.

Table 4: Categorization of rhizobacterial isolates based on proteolytic index (PI)

Plant	Number of rhizobacteria screened	Number of Non-producer	Number of protease producers		
			Proteolytic Index (PI)		
			Poor (PI < 1.4)	Moderate (PI 1.4-1.7)	Good (PI > 1.7)
***Mangifera indica* L.**	06	03	01	0	02
***Averrhoa carambola* L.**	05	0	01	02	02
***Areca catechu* L.**	05	0	0	04	01
***Cycas circinalis* L.**	11	01	04	05	01
TOTAL	27	04	06	11	06

Proteolytic Index was calculated based on growth on casein supplemented nutrient agar

4.5 Secondary screening for protease production in submerged fermentation

Six selected rhizobacteria (CGR01, CGR 04, SCA03, SCA04, SCB01 and SCC03) showing a proteolytic index > 1.7 were further screened for growth associated enzyme production in submerged fermentation in casein supplemented mineral salts medium. It was observed that growth as well total enzyme activity was significantly high after 24 h as compared to 48 h of incubation (Table 5). This may be attributed to a better growth performance of the bacteria even under limited nutrient availability in synthetic medium [16, 17]. Bacterium CGR04 from mango rhizosphere showed highest protease production which amounted to 275.4 U / ml after 24 h while it declined to 221.6 U / ml after 48 h. This isolate was further selected as potent protease producing bacterium for optimization studies.

4.6. Determination of taxonomic identity of potent isolate

The morphological, physiological and biochemical characteristics of the selected bacterial isolate CGR04 was determined using standard microbiological methods and compared with Bergey's Manual of Determinative Bacteriology [18]. The bacterium is Gram-positive, spore forming, rod-shaped producing catalase, amylase, gelatinase, cellulase, lysine decarboxylase, ornithine decarboxylase and nitrate reductase. The bacterium produced acid from arabinose, cellobiose, dextrose, fructose, mannitol, mannose, salicin, sorbitol and sucrose (Table 6).

Table 5: Secondary screening of rhizobacterial isolates for growth associated proteolytic activity in submerged fermentation

Rhizobacteria	Incubation, h			
	24		48	
	Growth, O.D. at 540 nm	Total Protease [a], U/ml	Growth, O.D. at 540 nm	Total Protease [a], U/ml
CGR01	1.18 ± 0.03	171.00 ± 8.73	0.94 ± 0.06	151.38 ± 7.9
CGR04	1.36 ± 0.0	275.40 ± 15.39	1.06 ± 0.18	195.66 ± 13.2
SCA03	1.26 ± 0.02	170.16 ± 10.6	0.87 ± 0.09	157.45 ± 9.59
SCA04	1.08 ± 0.03	162.50 ± 6.34	0.93 ± 0.01	142.88 ± 16.21
SCB01	1.18 ± 0.0	162.41 ± 12	0.91 ± 0.05	146.61 ± 3.7
SCC03	1.30 ± 0.0	200.82 ± 18.9	0.98 ± 0.0	172.13 ± 18.64

[a] Total protease production was assayed during growth of bacteria in casein supplemented mineral salts broth after 24 and 48h of incubation.
One unit (U) of protease was defined as the amount of enzyme that releases 1 µM of tyrosine per ml per h

The organism was found to be resistant to ampicillin but showed sensitive to other antibiotics. The bacterium was tentatively identified as *Bacillus* sp. CGR04. Several studies have mentioned that majority of the protease producers belong to the genera *Bacillus* [19, 20] and this report is also not a deviation.

4.7. Optimization of protease production during growth

Time course:

Growth associated protease production by *Bacillus* CGR04 was monitored in mineral salts medium containing 1% casein under submerged cultivation. Results (Fig 1) show that production of protease was more or less parallel with growth of the organism, which attained a peak (290.01U/ml) during the early stationary phase (24h) of growth suggesting that protease is a primary metabolite and its production occurred during trophophase. Similar reports showing optimal protease activity at 24 h of growth was observed in *Micrococcus aloeverae* AE-6 [21] and *Bacillus* sp. [22] and enzyme activity gradually declined during idiophase in both the organisms.

Table 6. Morphological and biochemical characterization of *Bacillus* CGR04

Characters	Response
Colony morphology	Cream, wrinkled, undulate, umbonate
Cell morphology	Rods in chain of 2-6 cells, rarely solitary, Size (l x b, µm) 2.8-4.2 x 0.7-1.4
Gram nature	Gram +ve
Endospore formation	+ central, elliptical
Diffusable pigment	None
Catalase	+
Amylase	+
Gelatinase	+
Cellulase	+
Citrate utilization	+
Lysine decarboxylase	+
Ornithine decarboxylase	+

Urease	-
Phenylalanine deamination	-
Nitrate reductase	+
H_2S production	-
***Fermentation of Sugar*:**	
Arabinose, Cellobiose, Dextrose, Fructose, Mannitol, Mannose, Salicine, Sorbitol, Sucrose	+
Adonitol, Galactose, Lactose, Maltose, Rhamnose	-
Antibiotic Sensitivity	Amp^r, Nov^i, Tet^i, Bac^s, Chl^s, $Chlt^s$, Cip^s, Ery^s, Kan^s, Rif^s, Str^s, Van^s

"+"=positive response; "-"= negative response,

Antibiotic sensitivity was assessed following Kirby Baur Disc diffusion method, r=Resistant, i=Intermediate, s=Sensitive

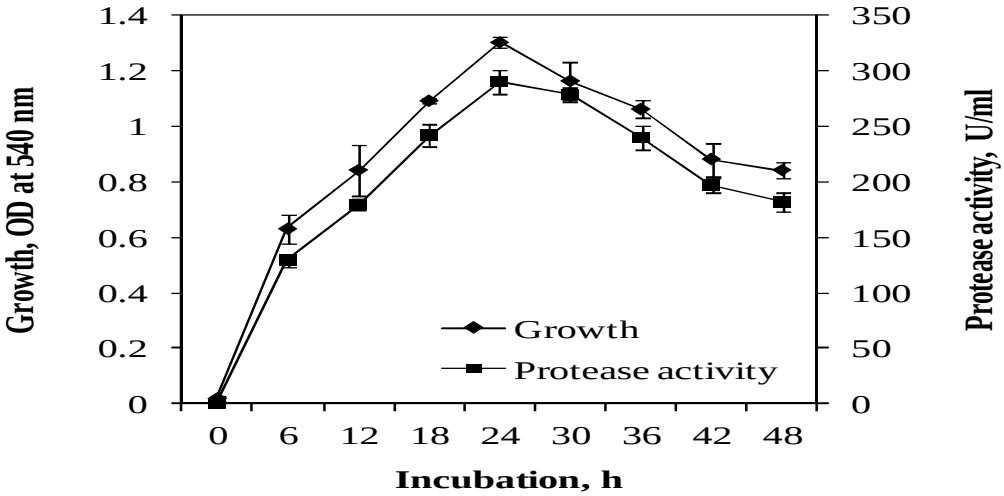

Fig 1. Time course of growth and extracellular protease production by *Bacillus* CGR04 in casein supplemented mineral salt medium. Protease production was assayed in mineral salts broth supplemented with casein. One unit (U) of protease was defined as the amount of enzyme that releases 1 µM of tyrosine per ml per h.

Effect of substrate concentration

Protease production during growth of the *Bacillus* CGR04 was found to increase with increasing concentration of substrate casein until 12.0 mg/ml (w/v) after 24 h growth and the maximum protease activity was found to be 335.86 U / ml (Fig 2A). However, growth as well as enzyme production declined at 1.5 %. The enzyme kinetics followed a linearized Lineweaver-Burk plot (Fig 2B) and the Km and Vmax of the enzyme were calculated as 4.77 mg/ml and 454.55 U/ml respectively. Casein has been previously reported as one of the ideal substrate for maximum protease yield in *Bacillus nakamurai* PL4 (0.4436 U / mL), *Bacillus subtilis* (168 U / mg) and *Bacillus cereus* AG1 (250 U / ml) respectively [23-25].

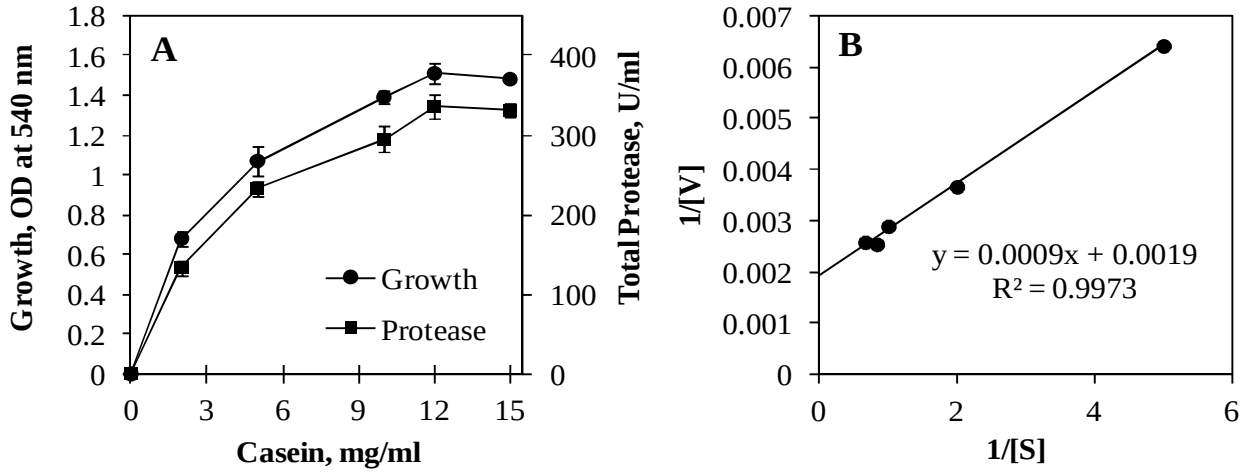

Fig 2. Effect of substrate concentration on the growth and protease produced by *Bacillus* CGR04 (A) and Lineweaver-Burk plot for showing enzyme kinetics (B). Protease production was assayed in mineral salts broth supplemented with casein. One unit (U) of protease was defined as the amount of enzyme that releases 1 µM of tyrosine per ml per h.

Effect of glucose and peptone concentration

The effect of carbon and nitrogen concentration on growth and protease production was measured using glucose and peptone as the carbon and nitrogen sources respectively (Fig 3). Protease production increased considerably from 1.0 – 2.5 % glucose and optimal enzyme activity amounted to 279.6 U / ml at 1 % level (Fig 3A). The mineral salts medium contain peptone as sole source of nitrogen and with increase in peptone concentration from 0.25 to 1.5 % in the growth medium, production of extracellular protease increased to 304.84 U / ml at 1 % level beyond which it declined significantly (Fig 3B). Bacterial isolates *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Exiguobacterium profundam* exhibited maximum protease activity and better growth performance ability in presence of glucose as C-source and peptone as the N_2- source in submerged fermentation [26, 27].

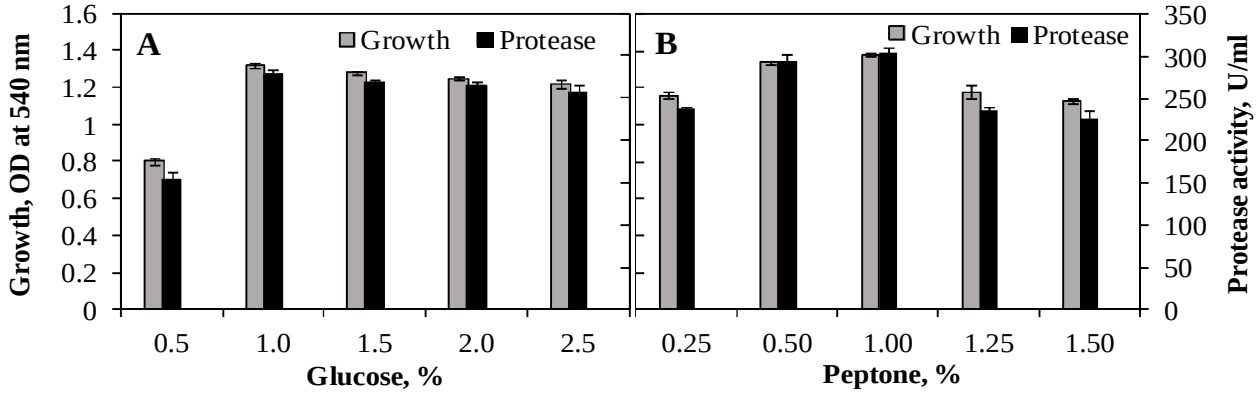

Fig 3. Effect of glucose (A) and peptone (B) concentration on growth and protease production by *Bacillus* CGR04. Protease production was assayed in mineral salts broth supplemented with casein. One unit (U) of protease was defined as the amount of enzyme that releases 1 µM of tyrosine per ml per h.

Effect of pH and temperature

Protease production is influenced by pH of the growth medium as well as growth temperature. In this study, *Bacillus* CGR04 growth of the bacterium was tested in the pH range of 6.0 to 8.0 and it was observed both growth and protease production optimized at slightly alkaline region (pH 7.5) and amounted to 307.10U / ml. The decline in protease production in acidic (6.0) and alkaline (8.0) pH proved that *Bacillus* CGR04 produced a neutral protease (Fig 4A). Although growth of the bacterium was optimal at a temperature range of 30-35ºC, but optimal protease yield was attained at 35ºC (297.3 U / ml). Our data coincided with that of Pant *et al*. [28] and Chaudhuri *et al*. [29] who reported neutral protease production at a pH of 7.4 and 30ºC in *Bacillus subtilis* and *B. aerius* UB02 respectively.

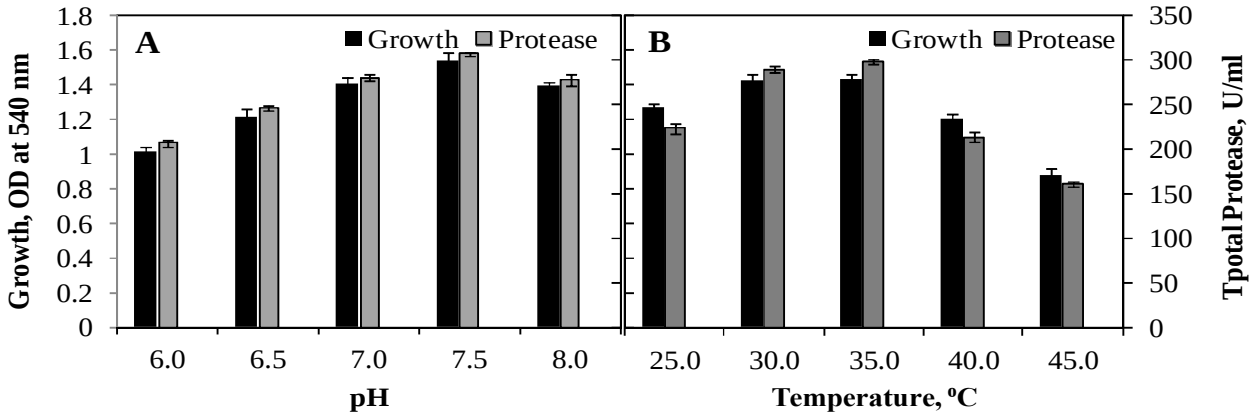

Fig 4. Effect of pH (A) and temperature (B) on growth and protease production by *Bacillus* CGR04. Protease production was assayed in mineral salts broth supplemented with casein. One unit (U) of protease was defined as the amount of enzyme that releases 1 µM of tyrosine per ml per h.

4.8. Conclusion

Bioprospecting of rhizospheric bacteria underneath commonly grown trees in the garden has shown that protease producers are not uncommon and few of them can produce neutral protease having benefits in food and brewing industry, particularly due to the fact that they generate less bitterness in hydrolyzing the proteinaceous compounds and are also insensitive to plant proteinase inhibitors. Furthermore, purification and characterization of the neutral protease from *Bacillus* CGR04 could be of potential significance for food as well as dairy processing applications.

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