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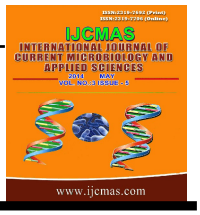

Original Research Article

Extracellular Protease Enzyme Production using *Micrococcus luteus-4, Staphylococcus hyicus , Micrococcus luteus-1, Pasteurella pneumotrop and Micrococcus sp.* isolated From Water Reservoirs

Sangeeta Kumari*

Amity Institute of Biotechnology, Amity University Haryana ,Gurgaon 122413, India
Corresponding author

K e y w o r d s

Protease, *Micrococcus luteus*-4, *Staphylococcus hyicus*, *Micrococcus luteus*-1,

A B S T R A C T

Five proteolytic bacteria isolated from three sites of Sagar lake were identified as Micrococcus luteus-4 (S-1A), Staphylococcus hyicus (S-2C), Micrococcus luteus-1 (S-4A), Pasteurella pneumotrop (S-3A) and Micrococcus sp.(S-5B). They were showed best enzymatic spectrum and optimum enzyme activity in pH range of 8-9 and temperature of 500C–600C. Soybean meal extract was the best nitrogen source for protease production whereas sucrose was the best carbon source. All the selected metal ions and inhibitors enhanced the enzyme production of all the selected bacterial isolates. Therefore, such enzymes considered as metallo proteases. For four isolates Staphylococcus hyicus, Micrococcus luteus-1 and Pasteurella pneumotrop and Micrococcus sp. metal ion CuSO4 increased the enzymatic activity at 1 mM whereas, in case of Micrococcus luteus-4 at 5 mM concentration.

Introduction

Proteases represent the class of enzyme, which occupy a pivotal position with respect to their physiological role as well as their commercial applications. They perform both degradative and synthetic functions. Microbes are attractive source of protease owing to the limited space required for their cultivation and their ready to susceptibility to genetic manipulation. These enzymes are important in a number of diverse and crucial biological processes; they are involved in the regulation of metabolism and gene expression, enzyme modification, pathogen city and the hydrolysis of large proteins to smaller molecules for transport and metabolism (Rao MB et al .1998).

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. They are essential constituents of all forms of life on earth, including prokoryates, fungi, plants and animals. They can be cultured in large quantities in relatively short time by established fermentation methods and produce an abundant, regular supply of the

desired product. In recent years there has been a phenomenal increase in the use of alkaline protease as industrial catalysts. Alkaline proteases (EC.3.4.21–24, 99) are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine center (serine protease) or are of metallo- type (metalloprotease); and the alkaline serine proteases are the most important group of enzymes so far exploited (Gupta et al., 2002). Microbial proteases are classified as acid, neutral and alkaline proteases on the basis of pH range in which their activity is optimum (Hankin L and Anagnostakis SC, 1976).

These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons; for example they exhibit high catalytic activity, a high degree of substrate specificity can be produced in large amounts and are economically viable. Microbial alkaline proteases dominate the worldwide enzyme market, accounting for two-third of the share of the detergent industry. Although production is inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially. Alkaline proteases of *Bacillus* sp. origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Agrawal et al. 2004). This paper deals with the isolation, identification and characterization of proteolytic bacteria form Sagar Lake, which may have potential application in various industries.

Materials and Methods

Isolation and maintenance of microbial strains

Water sample from three selected sites (Site A-Dhobhi ghat, Site B- Temple site, Site C- Chakra ghat) of the Sagar Lake in a presterilized 100 mL stoperred bottle. Samples kept in icebox and brought to the laboratory and processed within 1h as the rapid changes take place in the bacterial content. An increase in bacterial numbers is accelerated by an increase in temperature (Purva et al. 1998). Bacterial colonies were isolated by dilution plate technique on nutrient agar by (Madan M, Dhillon S and Singh R, 2000). The bacterial colonies were purified by repeated streaking on fresh medium and maintained at 40C on slants of nutrient agar containing 1% gelatin, which acts as an inducer for the production of protease enzymes.

Screening and identification

The bacterial strains were screened for proteolytic activity by growing on gelatin yeast extract agar (Jacob MB and Gerstein MJ, 1960) containing in gL-1: gelatine 10, glucose, 1; yeast extract, 0.2; CaCl2, 0.02; MgSO4. 7H2O, 0.5; K2HPO4, 0.3; KH2PO4, 0.1 and agar, 20 at 37°, 10°C. After 24h of incubation, the plates were flooded with 1% mercuric chloride. Colonies showing clear zone indicated the production of protease. Protease producing bacteria were tentatively identified on the basis of morphological, cultural and biochemical characteristics according to Berqey's Manual of Systematic Bacteriology (Sneath HAP and Halt GJ, 1986) and PIB computer kit, Bryant TN (1989).

Growth and protease production

Erlenmeyer flasks (250 ml) containing 50 ml production medium (Jacob MB and Gerstein MJ, 1960) were inoculated with 2% of 48h old inoculum of each bacterial isolate and incubated for 2-8 d at 400C in a rotatory shaker incubator (140 rpm), (Adinarayana K, Ellaiah P, and Prasad DS, 2003) and then centrifuged at 8000 rpm for 15 minute to obtain the cell free culture filtrate, which was used as the crude enzyme source. The protease activity was assayed by casein hydrolysis method4.The reaction mixture containing 1 ml casein solution, 0.5 ml of 0.05 M sodium citrate buffer and 0.5 ml of crude enzyme extract was incubated at 37° 10°C for 20 min. The reaction was stopped by adding 4 ml of 5% TCA. Liberation of tyrosine was determined at 660 nm against control prepared by adding TCA prior to addition of casein solution. One unit of enzyme activity represented the amount of enzyme that liberated mg mL-1 min-1 of tyrosine under the defined conditions. Protein content was estimated by the method (Lowry et al .1951) using bovine serum albumin as the standard.

Effect of different variable on protease production

The effects of key variables (pH, temperature and metal compounds, different nitrogen and carbon sources) on protease production were studied. The optimum Ph for the protease activity was determined by using buffer of pH ranging 4-11(Mcllvaine's; pH 4.0 - 8.0, and Borax-NaOH ; pH 9.0 - 12.0). Thermostability of protease was determined with a temperature range of 30-900C and also measuring the residual activity at different time intervals (I5-120 min.). Effect of various metal compounds(BaCl2., MgSO4, CuSO4, COCl2, CaCl2, EDTA, Mercuric chloride, Sodium azide) were determined by incubating the crude enzyme with 1 and 5 Mm concentration of each compound. Effect of various carbon sources (glucose, sucrose, lactose) and nitrogen sources (beef, tryptone, soybean meal) were determined by incorporating 1% of each source separately in the gelatin glucose yeast extract agar media (GGYA) All experiments were performed in triplicates.

Statistical Analysis

The results were subjected to statistical Analysis of Variance (ANOVA), using a Statistical Analysis Software (SPSS-13.0). The significant difference between means was determined by Duncan's Multiple Rang Test (DMRT), where p=0.05 was considered for significant difference.

Results and Discussion

35 were proteolytic among 135 bacterial isolates. Proteolytic activity among the isolates from different samples was found to vary (Table1&2). Further 10 bacterial isolates were selected for the present study on the basis of maximum inhibition zone and enzyme production. The maximum protease enzyme activity with *Pasteurella pneumotrop* followed by *Staphylococcus hyicus*, *Micrococcus* sp. *Micrococcus luteus-4* and *Micrococcus luteus-1*, while average protease activity was recorded with *Streptococcus* sp-1, *Streptococcus* sp-2 and *Pseudomonas*. Minimum protease activity was observed with *Xenorhahus luminescens* and *Micrococcus varians* on 4th d of incubation. Similar observations were reported by many workers (Ellaiah et al. 2002; Sandhia GS and Prema P, 1998) With regard to incubation period, all the bacterial isolates showed maximum activity on 4th day. After peak value there

was gradual decrease in activity, which may due to denaturation of enzymes with increased incubation period. Our findings are with the close agreement other workers (Purva et al.1998). The five isolates were further selected for detailed partial.

Characterization Studies

All the five selected bacterial isolates are alkaline in nature as they showed their activity upto pH 9 and there after declined (Fig 1). The statistical analysis revealed the mean value of enzyme activity was highest at pH=9 and in the temperature range of 55-60OC which depicts its thermo tolerance as well as alkaline nature at p=0.05 significance level (Table-5). Majority of organisms systhesized protease under alkaline conditions (Dhandapani R and Vijayaragavan R, 1994; Nehete PN, Shah VD and Kathari RM, 1986; Naidu KSB and Devi LK, 2005; Chaia et al. 2000).

Maximum enzyme activity for *Micrococcus luteus*-1 and M*icrococcus tuteus-4* was at 500C, whereas *Pasteurella pneamotrop Staphylococcus hyicus* and *Micrococcus* sp. was at 600C (Fig.2). Many workers were also reported similar findings (Olajuyigbe FM and Ajele JO, 2005; Park et al. 2003; Lin et al, 1997; Klingeberg M, Hashwa F and Antranikian G, 1991).

Among the carbon and nitrogen sources tested sucrose and soybean meal were found to support maximum protease activity of all the five bacterial isolates (Table3). Similar results have been recorded with *Bacillus* species Sen S and Satyanarayana T (1993). For three isolates *Micrococcus sp.*, *Staphylococcus hyicus* and *Micrococcus luteus-1*, the metal ions (CuSO4) and inhibitors (sodium azide, EDTA and HgCl2) showed increased activity at 1mM concentration and decreased activity at 5mM concentration. Some metal ions (CoCl2, CaCl2, BaCl2, MgSO4 and K2HPO4) showed increased activity at 5mM and decreased activity at 1mM concentration which shows that the concentration of the metal ions also influence the increase or decrease in enzyme activity (Table 4). Effect of various metal ions and known protease inhibitors were studied on the enzyme activity of *Aspergillus aryzae* NRRL 2217 Sumantha et al (2005). The effect of various protease inhibitors were studied on the activity of protease of *Bacillus subtilis* strain 38 and found that only 1, 10 – phenanthroline decreased the enzyme activity indicating the presence the metalloproteases Chantawannakul et al.(2002) Most of the tested metal ions (Ca 2+, Mg 2+ and Mn 2+) had a stimulatory effect or a slight inhibitory effect on enzyme activity. These cations have been reported to increase the thermal stability of other *Bacillus* alkaline proteases (Paliwal N, Singh SP and Garg SK, 1994; Rahman et al. 1994).

The effect of different inhibitors on the enzyme activity of the purified protease of *Bacillus subtilis* PE-11 was observed with various inhibitors at 5mM concentration. Phenylmethyl sulfonyl fluoride (PMSF) was able to inhibit the protease completely, while diisopropyl fluorophosphates (DFP) exhibited 94% inhibition. Metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperatures (Pan T and LinS, 1992).

There was no complete inhibition of enzyme by EDTA, Sodium azide and mercuric chloride in all the five strains. It

is reported that EDTA sometimes do not required for its catalytic functions Sinha N and Satyanarayana T (1991). It can be concluded from these results that all the five tentatively identified bacterial isolates were thermotolerant and appeared to produce metal ion dependent alkaline protease.

Table.1 Proteolytic activity of tentatively identified bacterial isolates on 1% gelatin glucose yeast extract agar (GGYA) medium

Isolate Number	Name of isolates	Proteolytic activity (zone diameter in cms)
S-1A	*Micrococcus luteus-4*	4.1
S-2A	*Xenorhabdus luminescens*	3.0
S-3A	*Pasteurella pneumotrop*	4.8
S-4A	*Micrococcus luteus-1*	4.3
S-6A	*Micrococcus varians*	3.1
S-8A	*Streptococcus* sp.	3.4
S-5B	*Micrococcus* sp.	4.7
S-7B	*Streptococcus* sp.	3.3
S-1C	*Pseudomonas mallei*	3.2
S-2C	*Staphylococcus hyicus*	4.4

S : Sagar lake

Table.2 Protease activity (EU/mL) of the ten bacterial isolates

Isolate No.	Name of Isolates	*Incubation period (d)*					*CD at 0.05%*
		0	**2**	**4**	**6**	**8**	
S-1A	*Micrococcus luteus-4*	0.003	0.038	0.063	0.049	0.024	**0.017**
S-2A	*Xenorhabdus luminescens*	0.001	0.041	0.040	0.021	0.017	**0.018**
S-3A	*Pasteurella pneumotrop*	0.002	0.037	0.076	0.038	0.021	**0.023**
S-4A	*Micrococcus luteus-1*	0.002	0.024	0.055	0.021	0.017	**0.013**
S-6A	*Micrococcus varians*	0.001	0.024	0.037	0.018	0.016	**0.016**
S-8A	*Streptococcus* sp.	0.001	0.033	0.046	0.019	0.018	**0.028**
S-5B	*Micrococcus* sp.	0.003	0.026	0.064	0.025	0.018	**0.017**
S-7B	*Streptococcus* sp.	0.001	0.028	0.048	0.026	0.017	**0.036**
S-1C	*Pseudomonas mallei*	0.001	0.031	0.042	0.022	0.017	**0.023**
S-2C	*Staphylococcus hyicus*	0.004	0.038	0.071	0.046	0.028	**0.035**

EU : mg/min/mL tyrosine liberated

Table-3: Effect of different carbon and nitrogen sources on protease activity (EU/mL) of bacterial isolate

Isolate No.	Name of Isolates	Carbon sources enzyme activity (EU/mL)			Nitrogen sources enzyme activity (EU/mL)			CD at 0.05%
		Sucrose	Lactose	Glucose	Soyabe anmeal	Beef	Tryptone	
S-1A	*Micrococcus luteus-4*	0.108	0.079	0.063	0.084	0.093	0.084	**0.057**
S-3A	*Pasteurella pneumotrop*	0.088	0.063	0.076	0.076	0.064	0.093	**0.052**
S-4A	*Micrococcus luteus-1*	0.119	0.103	0.065	0.129	0.123	0.110	**0.073**
S-5B	*Micrococcus* sp.	0.066	0.056	0.064	0.073	0.072	0.131	**0.042**
S-2C	*Staphylococcus hyicus*	0.076	0.116	0.071	0.061	0.084	0.110	**0.056**

EU : mg/min/mL tyrosine liberated

Table - 4 : Effect of metal ions and inhibitors on the protease activity (EU/mL) of bacterial isolates

Metals	Concen-trations (mM)	Isolates									
		Micrococcus luteus-4 (S-1A)		*Pasteurella pneumotrop* (S-3A)		*Micrococcus luteus-1* (S-4A)		*Micrococcus sp.* (S-5B)		*Staphylococcus hyicus* (S-2C)	
		EA	Effect (%)	EA	Effect (%)	EA	Effect (%)	EA	Effect (%)	EA	Effect (%)
$BaCl_2$	1	0.012	+ 42.85	0.020	+ 20.00	0.015	+ 37.50	0.014	+ 44.00	0.015	+ 28.57
	5	0.014	+ 33.33	0.017	+ 32.00	0.014	+ 41.67	0.021	+ 16.00	0.012	+ 42.85
$MgSO_4$	1	0.011	+ 47.62	0.015	+ 40.00	0.017	+ 27.16	0.017	+ 32.00	0.009	+ 57.14
	5	0.009	+ 57.14	0.014	+ 44.00	0.014	+ 41.67	0.013	+ 48.00	0.014	+ 33.33
K_2HPO_4	1	0.013	+ 38.09	0.021	+ 16.00	0.018	+ 25.00	0.011	+ 56.00	0.011	+ 47.62
	5	0.014	+ 33.33	0.019	+ 24.00	0.019	+ 20.83	0.018	+ 28.00	0.005	+ 64.00
$CuSO_4$	1	0.016	+ 23.81	0.017	+ 32.00	0.011	+ 54.17	0.019	+ 24.00	0.009	+ 57.14
	5	0.015	+ 28.57	0.013	+ 48.00	0.013	+ 45.83	0.021	+ 16.00	0.018	+ 14.28
$CoCl_2$	1	0.015	+ 28.57	0.013	+ 48.00	0.020	+ 16.67	0.013	+ 48.00	0.013	+ 38.09
	5	0.019	+ 9.52	0.009	+ 64.00	0.021	+ 12.50	0.016	+ 40.00	0.016	+ 23.81
$CaCl_2$	1	0.017	+ 19.05	0.018	+ 28.00	0.019	+ 20.83	0.019	+ 24.00	0.011	+ 47.62
	5	0.019	+ 09.52	0.013	+ 48.00	0.016	+ 33.33	0.017	+ 32.00	0.015	+ 28.57

Inhibitors											
Sodium azide	1	0.018	+ 14.28	0.020	+ 20.00	0.017	+ 29.17	0.018	+ 28.00	0.013	+ 38.09
	5	0.014	+ 33.33	0.014	+ 44.00	0.015	+ 37.50	0.019	+ 24.00	0.018	+ 14.28
EDTA	1	0.013	+ 38.09	0.018	+ 28.00	0.013	+ 45.83	0.017	+ 32.00	0.014	+ 33.33
	5	0.009	+ 57.14	0.016	+ 36.00	0.016	+ 33.33	0.016	+ 36.00	0.013	+ 38.09
$HgCl_2$	1	0.009	+ 57.14	0.016	+ 36.00	0.013	+ 45.83	0.018	+ 28.00	0.016	+ 23.81
	5	0.006	+ 71.42	0.011	+ 56.00	0.011	+ 54.17	0.013	+ 48.00	0.012	+ 42.85
Control (C)			**0.021**		**0.025**		**0.024**		**0.023**		**0.021**

EA : Enzyme activity.

Effect (%) : C-EA x 100/C

Compared to control increased activity = +

Table 5: Enzymatic Activity at various pH and Temperature profiles

pH range	Temperature range(^{O}C)	Enzymatic Activity
4	**30**	**0.057±0.01**
5	**35**	**0.063 ±0.01**
6	**40**	**0.027±0.00**
7	**45**	**0.037±0.00**
8	**50**	**0.072±0.00**
9	**55**	**0.100±0.039**
10	**60**	**0.085±0.00**
11	**65**	**0.355±0.26**

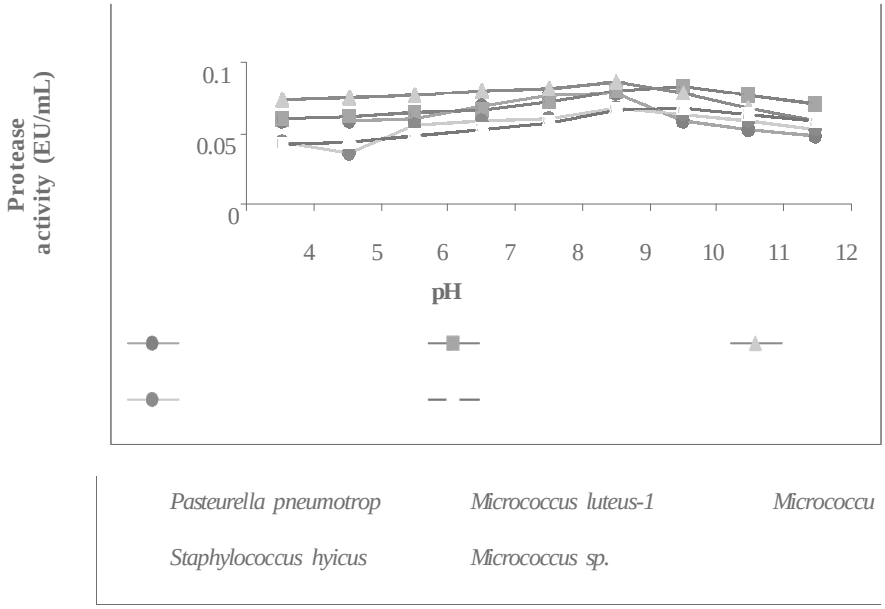

Fig 1: Effect of pH on protease activity (EU/ml) of five selected bacterial isolates

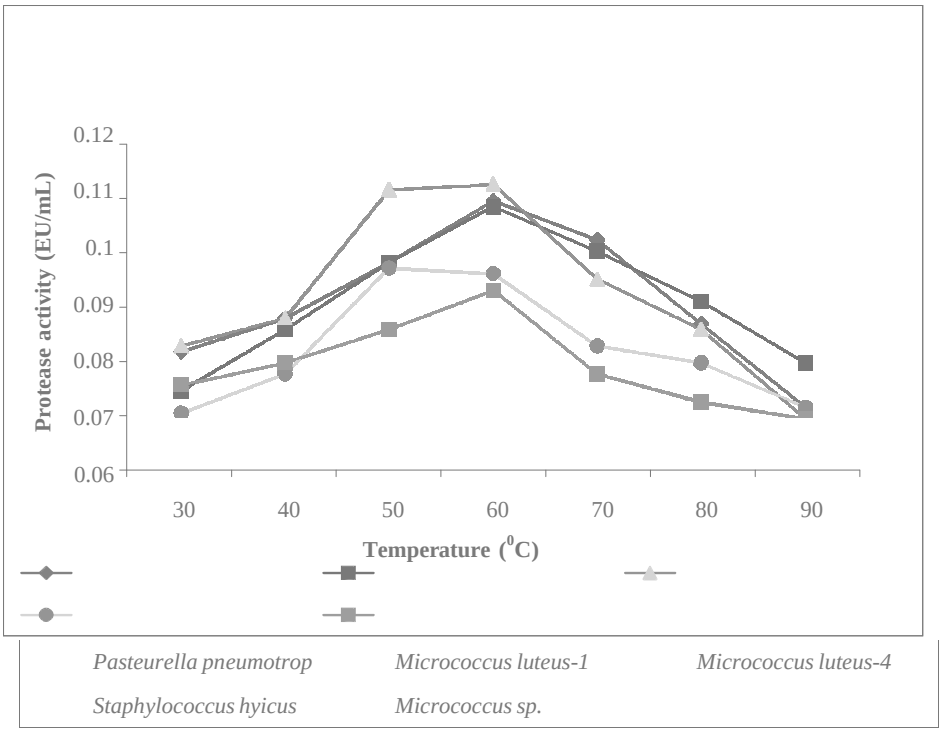

Fig.2 Effect of temperatureon protease activity (EU/ml) of five selected bacterial isolates

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