

Production of Protease from *Listeria monocytogenes*

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ABSTRACT

Listeria monocytogenes was isolated from degraded meat of cow and screened for proteolytic activity on the basis of its ability to hydrolyze the skimmed milk casein, gelatin and egg albumin. The isolate showed maximum protease production at 2 days of incubation time with medium pH 9.0 and 30°C temperature. (NH₄)₂HPO₄ and cellulose induced protease production when used as nitrogen and carbon sources respectively.

Key Words: *Listeria monocytogenes*; Protease

INTRODUCTION

Microbial proteases play an important role in biotechnological processes accounting for approximately 59% of the total enzyme used (Maugh, 1984). Proteases are very important group of industrial enzyme in many foods, clinical and tanning industry and in the manufacture of biological detergent (Aunstrup, 1980).

Proteases from *Aspergillus* (Cohen, 1977) have been reported as both intracellular (Holzer, 1975) and extracellular (Cohen, 1977; Haab, 1990; Rahman *et al.*, 1995). Proteases show variety of characteristics under different conditions. Microorganisms, which produced extracellular acid proteases, often acidify the medium in which they grow (Cohen, 1977; Matsushima *et al.*, 1981), and the ability to produce alkaline proteinases has been correlated with growth of organisms at neutral to alkaline proteases (Matsushima *et al.*, 1981). Formation of proteinases varies in the presence of different carbon and nitrogen sources (Micales, 1992; Rahman *et al.*, 1995; Shumi *et al.*, 2004), medium pH (Micales, 1992; Hossain *et al.*, 1999.), and also the incubation temperature and time (Marzan, 2002; Shumi *et al.*, 2004). Heat stable alkaline proteases, reported by many workers (Berla-Thangam & Suseela-Rajkumar, 2002), have potential for industrial use.

In this communication, we report the isolation and selection of a bacterium (S₁), which is a potent producer of extracellular protease, and the optimization of culture conditions required for enzyme production by the isolate.

MATERIALS AND METHODS

Isolation, purification, preservation and screening of microorganisms. The samples for the research work were taken from degraded meat of cow. Enrichment medium was used for isolation of protease producing microorganisms. The isolated organisms were then purified through repeated plating method in nutrient agar medium. Egg albumin degradation, skimmed milk casein hydrolysis, and gelatin

hydrolysis method were followed for primary screening of microbes and then an isolate was selected for the protease activity in liquid medium by quantitative method.

Quantitative method. For *in vitro* production of protease by the isolate, a liquid broth medium containing Tryptone 1%, Dextrose 0.1%, and yeast extract 0.5% (pH 6.5) was used (Matta *et al.*, 1997). After sterilization, the medium was inoculated at 37°C for 48 h. After incubation the culture filtrates were collected by centrifugation at 1000 x g for 12 minutes at 4°C. The supernatant was used as crude enzyme.

Measurement of enzyme activity. Enzyme assay was done by the modified method of Hayashi *et al.* (1967), as followed by Meyers and Ahearn (1977). The enzyme activity was expressed in units (U). One unit of enzyme was defined as the amount of enzyme that releases 1 µg of tyrosine per mL of crude extract per hour.

Biomass yield. Bacterial biomass was determined by measuring the absorbance at 600 nm (Henriette *et al.*, 1993).

Identification of selected isolate. Identification of selected isolate was studied based on different morphological, physiological and biochemical characteristics (Table I). The data was compared with standard description given in Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974).

Determination of culture conditions for maximum production of protease. To optimize the culture conditions for maximum production of protease different parameters such as incubation periods (24, 48, 72 and 96 h), medium pH (5.0, 6.0, 7.0, 8.0 and 9.0), temperature (30±2°C, 37±2°C, and 45±2°C), carbon sources (glucose, lactose, cellulose and cellulose) and nitrogen sources [peptone, tryptone, KNO₃ and (NH₄)₂HPO₄] were used. The effect of these parameters on protease production was recorded.

Determination of Optimum Conditions for the Crude Enzyme Activity

Enzyme-substrate reaction pH. The effect of pH on the protease activity was studied using phosphate buffer at pH ranged from 5.5 to 8.0 (Mahadevan & Sridhar, 1982).

Table I. Morphological, cultural and biochemical characteristics of the bacterial isolate S₁

Vegetative cells	Short rod or rod but coccoid in old culture, single. Size 1.3µm to 0.9µm (Fig. 4)							
Spore staining	Not formed							
Gram staining	Positive							
Acid fast staining	Not acid fast							
Motility test	Positive							
Agar colonies	Light yellow, circular, raised, entire, smooth colonies (Fig. 3)							
Agar slant	Filiform							
Nutrient broth	Turbid growth							
Catalase test	Positive							
Oxygen relationship	Aerobic							
Indole test	Not formed							
Nitrate reduction test	Positive							
Liquefaction of gelatin	Hydrolysed							
H ₂ S production	Positive							
Voges-Proskauer test	Negative							
Growth at different temperature	10 ^o C	27 ^o C	30 ^o C	37 ^o C	45 ^o C			
	-	++++	+++	++	-			
Growth at different NaCl concentration (%)	0	1	2	3	4	5	6	7
	++	++++	+++	+++	+++	++	-	-
	++							
Starch hydrolysis	Not hydrolysed							
Gelatin hydrolysis	Positive							
Urease test	Negative							
Oxidase test	Negative							
Fermentation of different carbohydrates	No change in Glucose, Fructose, Xylose, Arabinose, Raffinose, Inulin, Rhamnose, Mannitol, Lactose, Galactose, Starch, Sucrose, Glycerol, Cellulose							

Note: ++++ = Heavy growth; +++ = Moderate growth; ++ Scanty growth; - = No growth

Enzyme-substrate reaction temperature. The effect of temperature on the protease activity was also studied at different temperature such as 30, 35 and 40^oC.

Statistical analysis. Possible treatment differences among the temperature, pH or carbon/nitrogen source were explored by the analysis of variance (ANOVA) and Duncan multiple range test (DMRT) by running SPSS and Excel software. DMRT was used to find significant differences among treatments, if any.

RESULTS

Isolation and screening. Five composite samples were collected and by using enrichment technique a total of 25 microbial colonies were isolated on the basis of their cultural and morphological characteristics. The isolates were then purified, preserved and tested for their proteolytic ability. In preliminary screening the isolate S₁ showed better ability to degrade or hydrolyze egg albumin, skimmed milk casein (Fig. 1) and gelatin (Fig. 2). When the isolate S₁ was allowed to grow in a selected liquid medium (Matta *et al.*, 1997) it showed better activity and was finally selected for further studies.

Identification of the Isolate. The bacterial isolate S₁, was characterized on the basis of morphological, cultural and biochemical properties (Table I). All these properties were then compared with the standard characteristics described in Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974). The bacterial isolate S₁ was found to belong to the genus *Listeria* and it was provisionally identified as *Listeria monocytogenes* Pirie.

Effect of incubation period on protease production.

When the isolate was grown in liquid culture medium to determine optimum incubation time the study was carried out at 1 to 4 days incubation time and maximum enzyme formation (13.26 U/ml) was recorded with 2 days of incubation period (Fig. 5). The biomass yield was found increase with an increase in the incubation period. The highest biomass yield (6.57 absorbance at 600 nm) was recorded after 4 days of incubation period and different growth characteristics also observed with different incubation period.

Effect of incubation temperature and pH on protease production.

To ascertain optimum medium pH and temperature the present investigation is carried out at different medium pH ranged from 5.0 to 9.0 with different temperature such as 30, 37 and 45^oC.

Fig. 1. Casein hydrolysis by the isolate S₁**Fig. 2. Gelatin hydrolysis by the isolate S₁**

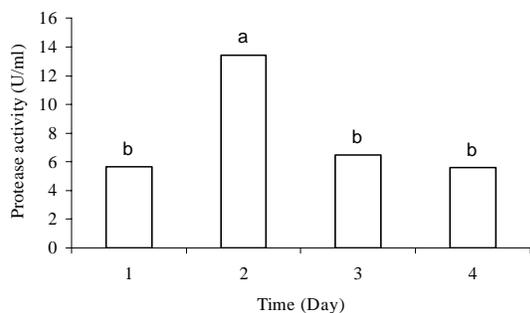
Fig. 3. Colony of the isolate on nutrient agar medium



Fig. 4. Microscopic feature showing negative staining of the isolate



Fig. 5. Effect of incubation period on production of protease by the isolate S₁



The same letters on the bar indicate no significant difference at $P < .05$ (ANOVA and DMRT).

The pH of the culture filtrate using isolate S₁ ranged from 6.4 to 7.8, 6.8 to 7.77 and 7.1 to 8.6 at 30, 37 and 45 °C respectively. Data suggested that the incubation temperature 30 °C and pH 9.0 is suitable for maximum protease

production by the isolate S₁, whereas the incubation temperature 30 °C and pH 7.0 is better for maximum production of biomass by the isolate S₁ (Table II). The growth characteristics and color of the supernatant varied at different pH and temperature ranges.

Effect of carbon and nitrogen sources on protease production. Maximum protease production was recorded with cellulose and (NH₄)₂HPO₄ when used as carbon and nitrogen sources in the medium respectively (Table III).

Effect of enzyme substrate reaction pH and temperature on crude protease activity. Table IV shows that the crude enzymes of S₁ showed maximum activity at 40 °C with reaction pH 6.5.

DISCUSSION

This study demonstrated that *L. monocytogenes* produced proteases at 2 days of incubation period with alkaline pH optimum. Extracellular protease production at alkaline pH by bacteria was also reported by Shin (1989), and Shalinisen and Satyararayana (1993).

The production of protease by *L. monocytogenes* in the presence of different carbon and nitrogen sources was variable. (NH₄)₂HPO₄ gave better enzyme secretion compared to other nitrogen sources studied here. Similar result were also obtained by Lee *et al.* (1992) while working on characteristic and action pattern of alkaline protease production from *Bacillus* sp. CW1121.

The crude enzyme of *L. monocytogenes* was found to be active at 40 °C, which are also in concurrence with the report of Lee *et al.* (1992).

CONCLUSION

The activity of protease from *L. monocytogenes* depends upon a number of factors including temperature, temperature and energy sources of the culture medium. The information on above factors will be useful in the future production protease(s) from *L. monocytogenes*.

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Table II. Protease production by the isolate S₁ under different temperature and medium pH

Incubation temperature	Protease activity (U/ml)					p
	Medium pH					
	5.0	6.0	7.0	8.0	9.0	
30°C	2.84±0.167 d	3.05±0.232 d	4.18±0.299 c	5.89±0.333 b	13.97±0.371 a	0.000
37°C	2.77±0.241 d	6.46±0.321 c	6.45±0.249 c	7.94±0.284 b	10.07±0.416 a	0.000
45°C	0.01±0.003 d	0.15±0.027 ab	0.17±0.021 a	0.10±0.013 bc	0.100±0.003 c	0.000

Note: Incubation time 2 days, Enzyme-substrate reaction temperature and pH 35°C and 5.5 respectively.

The same letter (s) in the row indicates no significant difference at $p < .05$ (ANOVA and DMRT).

Table III. Protease production by the isolate S₁ in different nitrogen and carbon sources

Nitrogen sources	Protease activity (U/ml)				P
	Carbon sources				
	Glucose	Lactose	Cellulose	Cellulose	
Peptone	5.6±0.272b	5.25±0.312b	6.10±0.311ab	6.81±0.216a	0.012
Tryptone	4.02±0.253 b	5.60±0.272a	3.97±0.22b	3.12±0.125c	0.000
KNO ₃	5.40±0.239 b	4.96±0.223b	5.39±0.248b	6.60±0.232a	0.002
(NH ₄) ₂ HPO ₄	6.17±0.266 b	5.89±0.198b	6.03±0.253b	7.80±0.216a	0.000

Note: Incubation time 2 days, Enzyme-substrate reaction temperature and pH 35°C and 5.5 respectively. Used Nitrogen source 1% and Carbon source 0.1%. The same letter (s) in the row indicates no significant difference at $p < .05$ (ANOVA and DMRT).

Table IV. Protease (crude) activity of the isolate S₁ under different pH and temperature

Enzyme-substrate reaction temperature	Protease activity (U/ml)					P
	Enzyme-substrate reaction pH					
	5.5	6.0	6.5	7.0	7.5	
30°C	3.37±0.085 d	2.61±0.031 e	5.67±0.085 a	4.8±0.041b	4.53±0.018 c	0.000
35°C	9.38±0.004 c	8.51±0.013 e	11.29±0.007 a	10.14±0.009 b	9.22±0.011 d	0.000
40°C	12.37±0.007 d	12.13±0.013 e	19.50±0.009 a	14.88±0.005 c	16.03±0.011 b	0.000
45°C	12.13±0.009 e	13.48±0.007d	13.75±0.015 c	14.47±0.009 b	14.89±0.013 a	0.000

Note: The same letter (s) in the row indicates no significant difference at $p < .05$ (ANOVA and DMRT).

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