INTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH AND BIOMEDICAL ANALYSIS | ISSN: 2278 –2664
| JULY-SEPT 2013| VOLUME 2 | ISSUE 3 | 14-20
Available Online Through: www.ijprba.com

Research Article

ENUMERATION OF BACTERIA PRODUCING THERMOTOLARANT PROTEASES FROM COMMERCIAL MILK SAMPLES OF WARANGAL (AP)

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ABSTRACT
Proteases are the proteolytic enzymes present in all forms of life. Several microbial strains including fungi and bacteria have reported to produce proteases. Among proteases microbial proteases secure most important place due to their enormous industrial applications. The present investigations are amide to enumerate the bacteria isolated from milk samples of commercial brands available in Warangal urban market to produce thermotalerant proteases. The incidence of proteolytic bacteria is common in all brands of milk samples. The thermotolarant proteolytic activity varied among the isolates studied. Casein was found to be good substrate than gelatin for the production of proteases. The present studies showed that maximum activity of thermotolerant proteases was expressed at 50^{o}C temperature.

KEY WORDS
Pasteurized milk, Bacillus sp, Thermotolerance, Proteases, Casein and Gelatin.

INTRODUCTION
Proteases are the class of enzymes which occupy key position with respect to their applications in both physiological and commercial fields (1). Today, proteases account for approximately 40% of the total enzyme sales in various industrial market sectors (2). The most important applications of protease are, used in laundry detergents, leather processing, brewing, food and pharmaceutical industries (3). The first detergent containing bacterial enzymes was introduced in the market in 1956 (4). Proteases, a group of enzymes hydrolyzing proteins into peptides and amino acids, are indispensable tools of all life forms including, viruses, bacteria, fungi, plants and animals. They have been associated with diverse functionality such as pathogenecity in irises (5). Hydrolysis of reserved protein during germination of plant seeds (6). Assimilation of proteins from environment, digestion of food in animals (7). Removal of unwanted proteins from the developing structures (8). Turning on of other proteins during a complicated physiological cascades (9). etc., among several. A large number of microbial strains including fungi (*Aspergillus flavus*, *Aspergillus miller*, *Aspergillus niger* and *pennicillium griseofulvum)* and bacteria *(Bacillus licheniformis, Bacillus firmus*, *Bacillus subtilis* and *Bacillus thuringiensis*) have reported to produce proteases. Although, various microorganisms were used to produce protease, the genus *Bacillus* have been so far the most important genus to produce proteases commercially (10). *Bacillus* species grows in pH range of 7.0-11.0 and produces extracellular proteases. Currently, a large proportion of commercially available alkaline proteases are derived from different strains of *Bacillus* (11). Microbial Protease production is highly influenced by media and metal ions (12, 13).

Besides this, several other factors, such as aeration/agitation, pH, temperature and incubation time etc also affect the amount of protease produced (14, 15). In the present investigation an attempt was made to enumerate the commercial branded milk samples for bacteria producing thermotolerant proteases.

MATERIAL AND METHODS

Milk sampling: Milk samples were collected at monthly intervals in the morning hours, during period from 2009 to 2011. Milk sachets were collected and transported to the laboratory in an icebox and stored at 4°C. The Samples were processed and analyzed within two hours of sampling. Different aspects of milk such as source, storage, transport, container, duration are carefully recorded.

Isolation & screening of *Bacillus* species producing thermotolerant protease from milk samples.

Selected samples were suspended and serial diluted with sterile distilled water. Each sample was heated at 50°C for 30 min in a water bath, spread on to Nutrient agar and subsequently incubated at 37°C for 24 hours. Colonies producing a clear zone were selected from LB supplemented with 2% skimmed milk agar and transferred onto a new agar plate by the inoculation technique following incubation at 50°C for 24hours. Bacteria having a clear zone diameter of more than 10 mm were selected for protease activity assay and were used throughout the study (16).

Assay of protease activity

The bacteria having a clear zone diameter of more than 10 mm were selected and cultivated in LB supplemented with 2 % skimmed milk broth. The culture broth was incubated at 50°C in a rotary shaker operated at 200 rpm for 24h. After words the bacterial cell cultures were centrifuged at 10,000 rpm for 10 min. The supernatant was collected and assayed for protease activity. The reaction mixtures containing 1 ml enzyme solution and 1ml of 1.5% casein were incubated in a water bath at 50°C for 10 min. 5ml of 0.4 M Na_2CO_3 and 2ml of Folin's reagent were added to terminate the reaction, and the reaction mixture was left to stand at room temperature for 10min. The protease activity was determined spectrophotometrically at 660nm. Additionally, the gelationolytic activity and caseinolytic activity were performed according to the above protocol using 1 % gelatin and 1 % casein, respectively.

Effect of temperature on protease stability: The effect of temperature on protease stability was evaluated by incubating at different temperatures i.e., 37°C, 50°C, 75°C, 100°C.

RESULTS AND DISCUSSION

In the present investigations, thermotolerant *bacillus* were isolated randomly from the milk samples of commercial brands and screened for protease enzyme production. All the protease producing bacterial isolates identified by primary screening were selected and among them 15 efficient strains were selected and their efficiency in producing the protease was assessed and the results are presented in **Table-1**. It is evident from the table that proteolytic potential of the isolates varied with the source of the isolation. Among all the isolates, BVM has shown maximum proteolytic potential. It was followed by BNM. Least proteolytic potential was exhibited by BMD.

The proteases produced by the present isolates were further characterized. In this regard the ability of the thermotolerant strains to produce proteases on two

different protein substrates that is 1% casein and 1% gelatin was evaluated and the results are presented in **Table-2**. It is evident from the table that protease production ability varied both with the isolate and substrate. It is obvious from the data that for all the isolates casein is the preferred substrate than the gelatin. It was almost double in casein substrate of all the isolates. BML secreted maximum enzyme using casein substrate. This strain is followed by BML. Least amount of enzyme was secreted by BWD. However, on gelatin the situation was different. On this substrate BVD produced maximum enzyme followed by BNM. The least secretion was made by BHT strain.

In continuation of studies on the activity of thermotolerant proteases produced by *Bacillus* speeches, an effort was made to observe the effect of temperature on specific thermotolerant proteases because temperature is one of the most critical parameters which controls bioprocessing (17). The results obtained in these studies are presented in **Table-3**. A critical study of the table reveals that protease activity of different isolates varied according to incubation temperature and also the isolate. However, in general, all the isolates have shown maximum activity at 50°C. Similarly Gouda (18) reported 60°C as optimum temperature for protease production by *Bacillus sp*. MIG. Next to 50°C almost all the isolates have shown more protease activity at 37°C. After 50°C temperature the specific protease activity decreased with increase in temperature and was least at 100°C. Maximum protease activity was shown by BVM, followed by BTM, BJR and BHT at 50°C. Least activity was shown by BMD, BPM, and BWD.

Table 1: Screening of *Bacillus sp* producing thermotolerant protease from milk supplied by different dairy farms.

S.No.	Isolate	Colony + zone diameter [mm]	Colony diameter [mm]	Zone diameter [mm]	Proteolytic potential
		[A]	[B]	[C]	
1	BAG	26.00	10.00	16.00	1.60
2	BDP	24.00	9.00	15.00	1.66
3	BHT	18.00	7.00	11.00	1.57
4	BJR	15.00	5.00	10.00	2.00
5	BMK	21.00	8.00	13.00	1.62
6	BML	14.00	4.00	10.00	2.50
7	BMD	21.00	10.00	11.00	1.10
8	BNM	13.00	3.00	10.00	3.33
9	BPM	25.00	11.00	14.00	1.27
10	BTM	25.00	7.00	18.00	2.57
11	BVD	14.00	4.00	10.00	2.50
12	BVM	12.00	2.00	10.00	5.00
13	BWD	26.00	10.00	16.00	1.60

BAG=*Bacillus from Agrigold,* ***BDP=*** *Bacillus from Dairypure,* ***BHT=****Bacillus from Heritage,* ***BJR=****Bacillus from Jercey,* ***BMK=****Bacillus from Mukunda,* ***BML=*** *Bacillus from Mulkanoor,* ***BMD=****Bacillus from Muthyms,* ***BNM=****Bacillus from Nagarjuna,* ***BPM=****Bacillus from Priya,* ***BTM=****Bacillus from Thirumala,* ***BVD=****Bacillus from Vijaya,* ***BVM=****Bacillus from Vyshnavi,* ***BWD=****Bacillus from Wardhannapet.*

Table 2: Proteolytic activity of thermotolerant *Bacillus sp* on 1% casein and 1% gelatin

S.No	Isolate	Protease activity [µg/mg protein]	
		1 % casein	1 % gelatin
1	BAG	1.049	0.567
2	BDP	1.030	0.635
3	BHT	1.058	0.408
4	BJR	1.064	0.657
5	BMK	1.098	0.581
6	BML	1.985	0.742
7	BMD	0.856	0.446
8	BNM	1.156	0.543
9	BPM	1.063	0.599
10	BTM	1.137	0.576
11	BVD	0.998	0.798
12	BVM	1.060	0.659
13	BWD	0.854	0.449

Table 3: Eeffect of temperature on thermotolerant protease activity of different isolates of Bacillus *sp.*

S.No	**Isloate**	**Thermotolerant protease activity [µg/mg protein]**			
		37°C	**50°C**	**75°C**	**100°C**
1	BAG	0.401	0.816	0.345	0.085
2	BDP	0.367	0.883	0.349	0.015
3	BHT	0.654	1.890	0.097	0.008
4	BJR	0.367	1.898	0.169	0.030
5	BMK	0.797	1.614	0.561	0.026
6	BML	0.743	1.034	0.401	0.087
7	BMD	0.118	0.383	0.010	0.009
8	BNM	0.937	1.934	0.630	0.010
9	BPM	0.192	0.394	0.102	0.082
10	BTM	0.564	1.982	0.198	0.067
11	BVD	0.425	0.926	0.202	0.020
12	BVM	1.609	2.083	0.404	0.038
13	BWD	0.208	0.402	0.149	0.042

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