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Production and partial purification of protease by selected bacterial strains using raw milk as substrate

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ABSTRACT

Aims: The present study was investigated to optimize and partially purify the proteases produced by the food borne bacterial strains.
Methodology and Results: Four bacterial strains such as *Bacillus cereus*, *Proteus vulgaris*, *P. mirabilis* and *Enterobacter aerogenes* were isolated from food wastes. These strains were individually inoculated in to the formulated culture media supplied with three different concentrations (1:1 to 1:3) of raw milk as major substrate. Among the concentrations, 1:2 ratio of substrate supplied medium showed maximum (0.133 to 8.000 IU/mL) protease production by all the tested organisms. After optimization, the organisms were tested for protease production at various pH (3 to 9), and temperature (30 to 80 °C). The result showed that all the organisms were capable of producing maximum protease at pH 6 (8.533 to 10.133 IU/mL) and at 50 °C (8.666 to 10.666 IU/mL). The crude enzymes produced by the tested organisms were individually purified by two different methods *viz* sodium alginate and ammonium sulphate-butanol methods. The purity of the protease determined in these two methods was ranged between 3.24 to 5.44 I and 3.13 to 5.55 IU/mL respectively. The partially purified enzymes were further analysed through SDS-PAGE; accordingly the molecular weight of protein produced by the test organisms was determined in between 49.44 and 50.98 kDa.
Conclusion, significance and impact of study: Among the tested strains *P. vulgaris* was identified as the major protease producer in optimized culture condition of 50^{o}C and pH6. The molecular mass of the partially purified protease of *P. vulgaris* was 50.32 KDa. Further research on optimization of other fermentation parameters using statistical tools with *P. vulgaris* is needed to scale up the process.

Keywords: Raw milk, Protease, *B. cereus*, *P. vulgaris*, *P. mirabilis*, *E. aerogenes*

INTRODUCTION

Protease catalyses the hydrolysis of proteins into peptides and amino acids, and consists of one of the most useful enzyme groups in both biochemical investigations and industrial applications (Kalisz, 1988; Rao *et al.*, 1998). Proteases are found in various microorganisms, plants and animals (Kumar *et al.*, 1999). Microbial proteases play an important role in biotechnological processes and they account for approximately 59% of the total enzymes used (Spinosaa, 2000). Proteases are having wide range of application in food, meat and leather processing industries, as well as pharmaceutical industries (Gupta, 2002). Several experiments conducted on microbes have been extensive production of protease in different culture conditions (Folasade and Ajele, 2005). Two *Lactobacillus* strains such as *L. homohiochii* and *L. curvatus* were isolated from Portuguese traditional dry formulated sausage showing high protease production, as well as these strains were having tyrene and ornithine decarboxylase activities (Pereir *et al.*, 2001).

The protease production mainly requires the appropriate substrates. There are many substrates used for protease production, which include skim milk, milk, peptone, casein etc. Some of the agricultural wastes, animal wastes, and plant wastes are also used as substrates for the production of protease, because they are readily available and economically very cheap and also they have high protein content. Yang *et al.* (1999) stated that whey is one of the good substrates used for protease production due to its high protein content. The experiments showed that the whey produced in dairies constituted a large amount of protein and consequently the study of its utilization by fermentation process could be of greater significance (Romero *et al.*, 1998). Milk is an ideal habitat for the growth and multiplication of microorganisms due to its nutritional constituents, which contain protein, carbohydrate, mineral and vitamins. All these components support the growth of many forms of

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bacteria (Frazier, 1967).Beside that, milk is an ideal medium for the growth of microorganisms from surrounding environment (Cousins and Barmley, 1981). Some of the psychrophilic bacteria present in milk synthesize sufficient amount of protease and helps preventing milk and milk products under refrigerated condition (Reilly and Day, 1983). Al-Saleh *et al.* (1997) stated that the *Pseudomonas flourescens* which was isolated from camel raw milk showed maximum growth and protease production in Trypticase soya broth. Considering the importance of milk, in the present study, an attempt was made to utilize raw cow milk as substrate for the production of extracellular protease by four different bacterial strains.

MATERIALS AND METHODS

Sample collection, isolation and identification of protease-producing strains

Three different samples such as slaughter meat waste, spoiled prawn and spoiled fish were aseptically collected from appropriate sites. The samples were homogenized individually and serially diluted from 10^{-1} to 10^{-8} by using distilled water. The serially diluted samples were inoculated in to Zobell Marine Agar plates and were incubated at 37 °C for 24 h. After incubation, the plates were observed for the growth of bacterial colonies. The individual colonies were further tested for protease activity by using dialyzed Brain Heart Infusion (D-BHI) milk medium in Petri plates (Pamela *et al.*, 1979), and the plates were incubated for 24 h at 37 °C. After incubation, apparent zone of hydrolysis on the D-BHI milk medium was observed around certain bacterial colonies. These protease producing colonies were further characterized by performing various morphological, physiological and biochemical tests described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1996), accordingly four bacterial strains such as *Bacillus cereus*, *Proteaus vulgaris*, *Proteaus mirabilis* and *Enterobacter aerogenes* were identified.

The protease activity in the liquid medium was assessed first, by culturing the individual bacterial strains, in an enrichment medium containing beef extract (0.3%), peptone (0.5%), NaCl (0.5%), and glucose (0.5%) at pH 7 for 24 h, and then 10% of enriched culture was inoculated in a 250-mL flask containing 45 mL basal medium containing (g/L): $(NH_4)_2SO_4$ 2 g, K_2HPO_4 1 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.4 g, $MnSO_4 \cdot H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, yeast extract 1 g, and peptone 10 g at pH 7. The culture was then incubated for 2 days by reciprocal shaking at 32 °C. The cells were then harvested by centrifugation at 10,000 x *g* for 15 min, and the supernatant was used for further protease assay.

Protease assay

The assay system consists of the following ingredients such as 1.25 mL Tris buffer (pH 7.2), 0.5 mL of 1% aqueous casein solution and 0.25 mL culture supernatant. Approximate controls were also made. The mixture was incubated for 30 min at 30 °C. Then, 3 mL of 5% tricarboxylic acid was added to this mixture, a precipitate was formed and it was placed at 4 °C for 10 min. Then, it was centrifuged at 5,000 x *g* for 15 min. From this, 0.5 mL supernatant was taken; to this 2.5 mL of 0.5 M sodium carbonate was added, mixed well and the mixture was incubated for 20 min. Then, it was added with 0.5 mL of folin phenol reagent, and the absorbance was read at 660 nm using UV-Vis Spectrophotometer (TECOMP 8500). The amount of protease produced was measured with the help of a tyrosine standard graph. Based on the tyrosine released, the protease activity was expressed in microgram of tyrosine released under standard assay conditions (Genckal and Tari, 2006).

Preparation and optimization of medium for protease production

The production medium was formulated with three different concentrations (1:1, 1:2 and 1:3 ratios) of sterilized raw cow milk obtained from local market and mixed at different radios of raw milk substrate with 0.2% yeast extract, 0.3% beef extract and 0.5% peptone. The media were prepared in sterilized conical flasks. After sterilization, the identified bacterial strains were individually inoculated into the respective medium separately and incubated under shaker incubator at 37 °C for 72 h. The protease production was measured at every 12 h interval during fermentation. Accordingly 1:2 ratio of raw milk supplied medium showed maximum protease production by majority of the test organisms. Therefore 1:2 ratio of raw milk added medium was taken and studied further to determine the effect of different pH (3, 4, 5, 6, 7, 8 and 9) and temperature (30, 40, 50, 60, 70, and 80 °C) optimization on protease production by individual test organisms.

Enzyme purification

The protease produced in the optimized culture conditions by the individual organisms was purified by two different methods *viz* Sodium alginate (or) Macroaffinity ligand-facilitated three-phase partitioning (MLFTPP) method followed by Kalyani *et al.* (2003) and Ammonium sulphate-butanol precipitation (ASBP) method by Bakare *et al.* (2005).

MLFTPP method of purification

Known volume of protease was mixed with 1 mL alginate (0.5% w/v) and the final volume was made up to 2 mL with 0.05M acetate buffer (pH 5). The enzyme containing solution was made up to 20% (w/v) with respect to ammonium sulfate by adding solid ammonium sulfate and vortexed the system. This was followed by addition of 2 mL of t-butanol to the above solution and vortexed gently, followed by incubating the systems at 37 °C for 1 h. After incubation, three phases (upper organic phase, interfacial precipitate, and lower aqueous phase) were observed.

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The above condition was known to precipitate alginate in a quantitative fashion in the interfacial phase. The upper t-butanol layer was removed carefully with a Pasteur pipette. After this, the lower aqueous layer was removed by piercing the interfacial precipitate layer using another Pasteur pipette. The difference between the total enzyme activity in the crude extract and the activity in the aqueous phase represented the amount of enzyme bound to the alginate in the interfacial layer. The interfacial precipitate consisting of alginate bound enzyme was dissolved in 3 mL of 1 M maltose and incubated at 4 °C for 4 h. Enzyme was then recovered by precipitating the alginate with 0.21 mL of 1M $CaCl_2$ (the final concentration of $CaCl_2$ in the solution was 0.07 M). Protease activity and protein in the supernatant were determined after extensive dialysis to remove maltose.

Ammonium sulphate-butanol precipitation (ASBP) method

Ammonium sulfate was added to the cell free culture suspension up to 70% of its saturation. The suspension was centrifuged at 16000 rpm at 4 °C for 15 min. The pellet was resuspended in 20 mM Tris-HCl buffer (pH 8.5) and dialyzed overnight against the same buffer with changes of buffer at least 3 times. The dialyzed supernatant was added to DEAE sephacryl matrix (2.0 mL), which was equilibrated with 30 mL of 20 mM Tris-HCl buffer (pH 8.5). They were shaken in an orbital shaker for 1 h at room temperature. The supernatant and the matrix were separated after the filtration under vacuum. The matrix was washed with 5 aliquots of 2 mL of 20 mM Tris-HCl buffer (pH 8.5). The proteolytic fractions were combined and were subjected to hydrophobic interaction chromatography. Then the pooled fractions from DEAE sephacryl fractionation were applied to a phenyl sepharose column which was equilibrated with 20 mM Tris-HCl buffer (pH 8.5) containing 2.5 M sodium chloride. The liquid was collected in a receiving tube. The column was washed with 20 mM Tris-HCl buffer (pH 8.5) and then bound proteins were eluted with 50% ethylene glycol in 20 mM Tris-HCl buffer (pH 8.5). Fractions were collected and then analyzed for protease activity. Fractions with highest enzyme activity were pooled and used for further characterization.

Protein determination

The protein content of supernatant as well as enzyme fractions obtained from the respective methods of MLFTPP and ASBP was determined by the method of Bradford (1976) using Bio-Rad dye reagent concentrate with BSA as the standard. The protein concentration was estimated by measuring the absorbance at 595 nm.

The specific activity and the purification factor of the enzyme produced were calculated by the following formula.

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity (U/mL)}}{\text{Total protein (mg/mL)}}$$

$$\text{Purification factor (U/mL)} = \frac{\text{Specific activity of purified enzyme}}{\text{Specific activity of crude enzyme}}$$

Determination of molecular mass of enzyme

Among the tested enzyme purification methods, the purity of protease was comparatively more in MLFTPP method. Therefore the molecular mass of the partially purified enzyme obtained through MLFTPP method was further determined by loading of 20 µg of partially purified protein in 10 % SDS-PAGE according to the method described by Laemmli (1970). The Rf value of the enzyme fraction was determined by referring the molecular mass of the markers using a gel documentation system (Syngene, UK).

Statistical analysis

The results obtained in the present study were subjected to relevant statistical analysis using Microsoft Excel 2005. Test for significant difference was analyzed using One-way analysis of Variance (ANOVA).

RESULTS

Identification of protease producing bacterial strains

Ten suspected isolates were taken from the meat slaughter waste, fish muscle and prawn muscle extracts and were subjected to various morphological, physiological and biochemical tests for identification purpose. Based on the results, four strains were identified as *B. cereus, P. vulgaris*, *P. mirabilis* and *E. aerogenes*. Among the four strains, three strains were Gram negative except *B. cereus*. All the strains represented positive in motility and also positive in catalyze, gelatin and glucose hydrolysis. Similarly, all the strains were negative in oxidase test. The MR/VP reactions were varied among the strains, accordingly *Protease* sp. was positive in MR/VP reactions and the other two strains were negative for the same reactions. The other physiological characteristics and also the specific carbohydrate utilization reactions were varied much among the strains (Table 1). All these four strains were further tested individually for protease production.

Protease production at various concentrations of substrates

The protease production by the candidate strains with different substrate concentrations during 12 to 72 h of incubation was assessed. The production level was varied much among the tested substrate concentrations. In all the tested strains, the protease production was maximum (0.133 to 8.000 IU/mL) at 1:2 ratio of substrate concentration within 24 to 36 h of incubation (Figures 1 and 4). Among the tested strains, the maximum protease

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production of 8.000 IU/mL was recorded by *B. cereus* at 24 h. When the substrate concentration level further increased to 1:3 ratio, the protease production level correspondingly reduced at all the tested organisms. For instance at 1:3 ratio substrate concentration, the minimum level of protease (2.133 to 7.066 IU/mL) production recorded in all the tested organisms, and among the tested organisms, *E. aerogenes* showed the lowest level of protease production with 2.133 IU/mL.

Table 1: Morphological, physiological and biochemical characteristics of isolated bacterial strains

Biochemical Characterization	*B. cereus*	*P. vulgaris*	*P. mirabilis*	*E. aerogenes*
Gram Staining	+	-	-	-
Motility	+	+	+	+
Indole	-	+	-	-
Methyl Red	-	+	+	-
Vogues Proskauer	+	-	-	+
Citrate	-	-	D	+
Urease	+	+	+	-
Catalase	+	+	+	+
Oxidase	-	-	-	-
H_2S	-	+	+	-
TSI	A/K	A/K	A/K	A
Nitrate	+	-	-	+
Casein	+	-	-	ND
Starch	+	-	-	-
Gelatin	+	+	+	+
Glucose	+	+	+	+
Fructose	+	-	-	+
Xylose	ND	+	+	+
Arabinose	-	-	-	+
Salicin	+	+	+	+
Mannose	+	-	-	-
Mannitol	-	-	-	-
Inositol	ND	-	-	+
Lactose	+	-	-	+
Sorbitol	ND	+	+	+
Sucrose	D	-	(+)	+
ODC	ND	+	+	+
LDC	ND	-	-	+
ADC	ND	-	-	-

+: Positive; **-**: Negative; (+): slowly positive; D: Dought full; ND: Not Done; A/K: Acid & alkaline; A: Acid; ODC: Ornthine decarboxlyase; LDC: Lysine decarboxlyase; ADC: Arginine decarboxlyase.

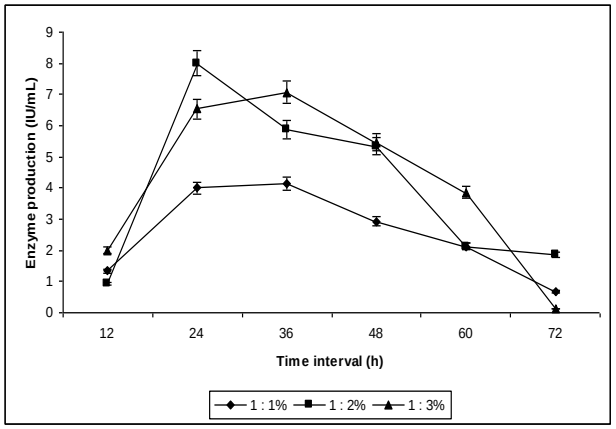

Figure1: Protease production (IU/mL) by *B. cereus* on different concentrations of milk broth (1:1 to 1:3) at different time intervals (12-72 h) of incubation.

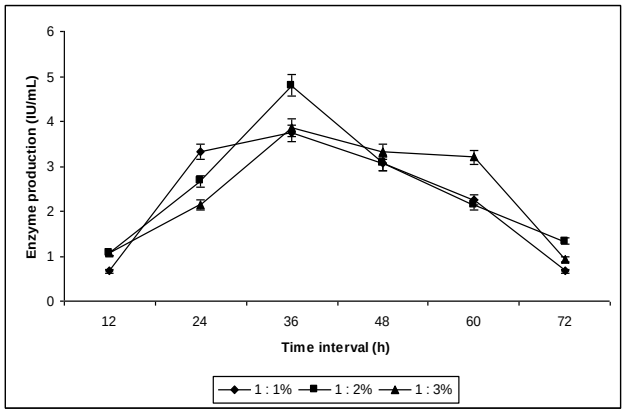

Figure 2: Protease production (IU/mL) by *P. vulgaris* on different concentrations of milk broth (1:1 to 1:3) at different time intervals (12-72 h) of incubation.

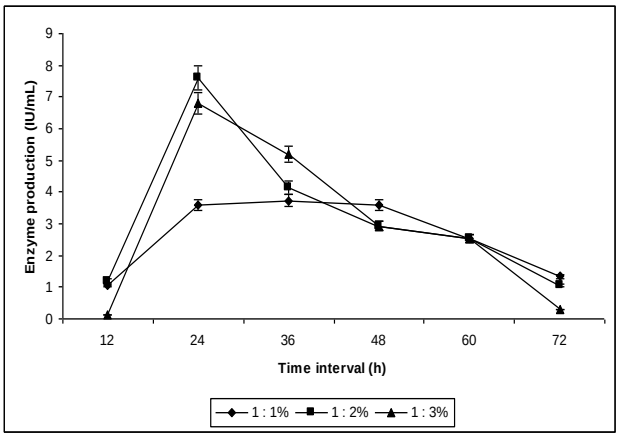

Figure 3: Protease production (IU/mL) by *P. mirabilis* on different concentrations of milk broth (1:1 to 1:3) at different time intervals (12-72 h) of incubation.

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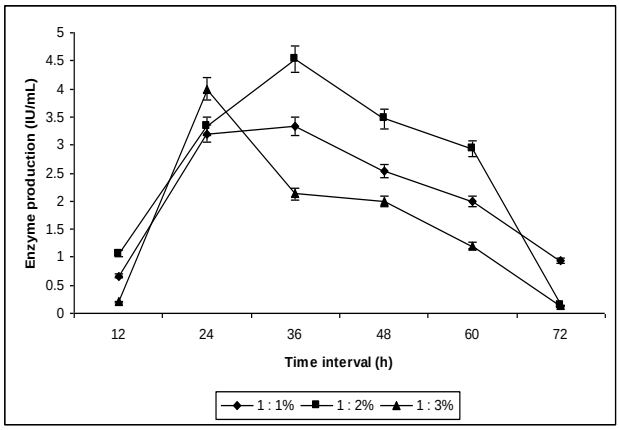

Figure 4: Protease production (IU/mL) by *E. aerogenes* on different concentrations of milk broth (1:1 to 1:3) at different time intervals (12-72 h) of incubation.

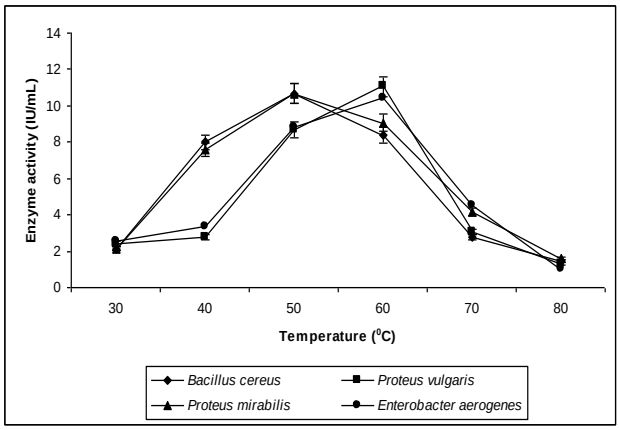

Figure 5: Protease activity (IU/mL) by different bacterial strains on 1:2 ratio of milk broth at different temperature (30-80 °C).

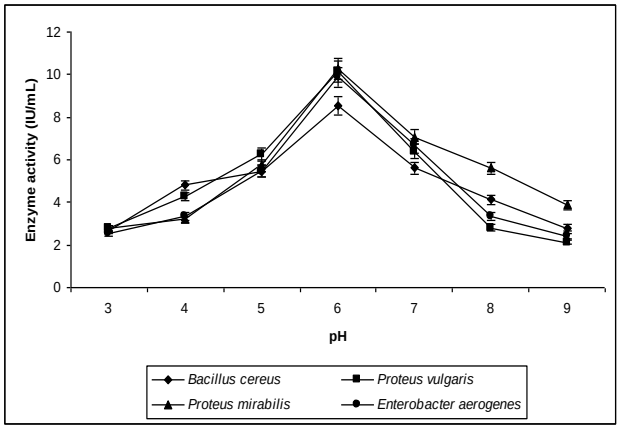

Figure 6: Protease activity (IU/mL) by different bacterial strains on 1:2 ratio of milk broth at different pH (3-9).

Protease production at various temperatures and pH

The optimized culture medium supplied with 1:2 ratio of substrate was further tested at different temperature and pH. The protease production recorded by the test organisms at various temperatures (30 to 80 °C) showed that maximum protease production was observed at 50 °C by all the tested organisms. Among the tested organisms *B. cereus* (10.666 IU/mL) produced maximum protease, whereas *P. vulgaris* produced minimum (8.666 IU/mL) amount. When the temperature level increased further, the protease production decreased significantly ($p< 0.05$) i.e. from 10.400 to 1.000 IU/mL by *E. aerogenes*, 9.066 to 1.600 IU/mL by *P. mirabilis*, 11.066 to 1.333 IU/mL by *P. vulgaris* and 8.400 to 1.466 IU/mL by *B. cereus* at 60 to 80 °C (Figure 5).

Similarly, the protease production recorded at different pH levels revealed that pH 6 was optimum for maximum protease production by all the tested organisms. Among these, *P. mirabilis* produced maximum of 10.266 IU/mL protease, followed by *P. vulgaris* (10.133 IU/mL), *E. aerogenes* (9.866 IU/mL) and *B. cereus* (8.533 IU/mL). When the pH level increased from 7 to 9, the enzyme production significantly ($p<0.05$) reduced in all the tested bacterial strains. It was from 5.600 to 2.800, 6.400 to 2.133, 7.066 to 3.866 and 6.666 to 2.400 IU/mL by *B. cereus, P. vulgaris, P. mirabilis* and *E. aerogenes* respectively (Figure 6).

Purification of protease

The crude enzymes produced by various test bacteria grown in media containing 1:2 ratio raw milk was individually purified by two different methods: viz. sodium alginate purification method as well as ammonium sulphate–butanol purification method, the result obtained is presented in Table 2.

Sodium alginate method (MLFTPP)

The protein content of crude enzyme of different test organisms grown in 1:2 ratio of raw milk substrate supplemented media showed more or less uniform result (2.16 to 2.6 mg/mL), whereas it was 0.94 to 1.08 mg/mL in the enzyme samples of various test organisms purified by sodium alginate method. After this purification process, 52.77 to 63.84 % of protein content was purified. The enzyme activity of purified samples of the tested organisms was ranged from 6.66 to 9.20 IU/mL, which was 38.75 to 130.0% more than that of enzyme activity of crude samples. Similarly the specific activity of purified samples of different test organisms was in between 6.172 and 9.219 U/mg. The increase in percentage of specific activity of all the tested organisms was 224.15 to 444.85%. The purification factor of the samples was in between 3.24 to 5.44 IU/mL.

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Ammonium sulphate butanol precipitation (ASBP) method

The protein content of enzyme samples of various test organisms purified by this method was from 0.880 to 1.040 mg/mL. After this purification process 52.38 to 66.15% protein content was purified. The enzyme activity of purified sample from tested organisms was ranged from 6.266 to 8.266 IU/mL, which was 30.54 to 87.86% more than that of enzyme activity of crude samples. Similarly the specific activity of purified samples of the tested organisms was ranged from 6.025 to 9.393 U/mL. The increase in percentage of specific activity of all the tested organisms was 213.54 to 455.14%. The purification factor of the samples was in between 3.135 to 5.551 IU/mL (Table 2).

Table 2: Protease activity (IU/mL), specific activity, protein content and purification factor of crude enzyme as well as different methods of precipitated enzyme produced by various bacterial strains on 1: 2 ratio milk broth substrates

Strain	Enzyme activity (IU/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Purification factor (IU/mL)
		Crude		
B. cereus	4.000	2.160	1.851	*
P. vulgaris	4.800	2.520	1.904	*
P. mirabilis	4.466	2.100	2.126	*
E. aerogenes	4.400	2.600	1.692	*
		Sodium alginate (MLFTPP) method		
B. cereus	9.200 (130.00%)	1.020 (52.77%)	9.019 (386.76%)	4.873
P. vulgaris	6.666 (38.75%)	1.080 (57.14%)	6.172 (224.15%)	3.240
P. mirabilis	7.733 (73.15%)	0.960 (54.28%)	8.055 (278.88%)	3.788
E. aerogenes	8.666 (96.95%)	0.940 (63.84%)	9.219 (444.85%)	5.448
		Ammonium sulphate – butanol (ASBP) method		
B. cereus	7.466 (86.65%)	1.020 (52.77%)	7.319 (295.40%)	3.954
P. vulgaris	6.266 (30.54%)	1.040 (58.73%)	6.025 (216.43%)	3.164
P. mirabilis	6.666 (49.26%)	1.000 (52.38%)	6.666 (213.54%)	3.135
E. aerogenes	8.266 (87.86%)	0.880 (66.15%)	9.393 (455.14%)	5.551

*Purification factor for crude enzyme; Values in parenthesis indicated percentage increase of enzyme activity, protein content and specific activity than the respective crude; Each value is a mean is ± SD of triplicate analysis.

Electrophoresis studies (PAGE analysis)

The polyacrylamide gel electrophoresis (PAGE) analysis of protease production of all the test organisms grown in 1:2 ratio of raw milk substrate was determined. The protein fractions were determined by densitometric scanning. The relative mobilities of protein fractions (Rf value) and corresponding molecular weight of each sample was determined (Figure 7). Accordingly the molecular weight of the identified protein fractions of *B.cereus*, *P. vulgaris*, *P. mirabilis* and *E. aerogenes* was 49.44, 50.32, 50.98 and 50.72 kDa, respectively.

DISCUSSION

Microbes represent an excellent source of enzymes including protease, because of their broad biochemical diversity (Godfrey and West; 1996). Microbial proteases are an important group of enzymes that can have application in various industries (Gupta *et al.*, 2002; Najafi and Deobagkar, 2005). Milk contains too little free amino acids and small peptides for sufficient growth of microorganisms, however, it contains 3-3.5% casein which can be degraded by proteinases and peptidases. Synthesis of proteolytic enzyme is therefore an essential requirement for good growth in milk (Fox, 1981). The experimental studies reported by Folasade and Ajele (2005) indicated that the proteases produced by nine *Bacillus* strains on 10% Skim milk agar plates. Similarly, *Pseudomonas* strain was used to produce protease in M.S. medium (Gaillard *et al.*, 2005), Reilly and Day (1983) also confirmed that the production of protease by using the bacterial strain *Aeromonas hydrophilla*. David and Mortimer (1977) stated that yeast *Saccharomyces cerevisiae* was used to produce protease enzyme. In the present study, four different bacterial strains were used for

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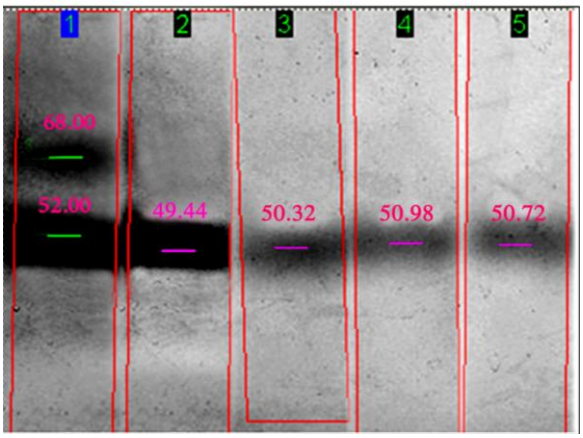

Figure 7: Analysis of protease enzyme fraction of selected test organisms on SDS-PAGE. Lane 1: Marker; Lane 2: *B. cereus*; Lane 3: *P. vulgaris*; Lane 4: *P. mirabilis*; Lane 5: *E. aerogenes*.

the production of protease using raw milk as substrate with three different concentrations (1:1 to 1:3 ratios). Among the test concentrations, maximum (4.0 to 8.0 IU/mL) protease production was recorded at 1:2 ratio of raw milk supplied medium by the tested organisms. The result particularly showed that maximum protease was produced by *B. cereus*, when compared with other strains. Past years, several researchers have performed the proteolytic enzyme production, for examples Miyaji *et al.* (2005) stated that extracellular protease was produced by *Stenotrophomonas maltophilia* S-1 in casein degrading medium. Johnvesly and Naik (2001) pointed out that the termophilic alkaline protease was produced by termophilic and alkaliphilic *Bacillus* sp. JB 99 in chemically defined medium. Similarly, Gaillard *et al.* (2005) pointed out the proteolytic activity of different *Pseudomonas* strains in raw milk supplemented medium.

The environmental factors such as pH and temperature are mostly influencing the enzyme production. In the present study also, the candidate strains were tested for their maximum ability on protease production in various level of pH and temperature, accordingly, we found that pH 6 and 50 °C temperature were optimum for maximum protease production by all the tested strains. Invariably, Chang *et al.* (2004) pointed out that pH 10 was optimum for high alkaline protease production by *Bacillus* sp, whereas Esakkiraj *et al.* (2007) reported that pH 7 and 60 °C temperature were optimum for maximum protease production by *B. cereus,* when it was grown at the substrate tuna fish waste supplied medium. Patel *et al.* (2005) was recorded that optimum pH for high protease production by haloalkalophilic *Bacillus* sp., when grown at gelatin broth medium at pH 8 and 9. Johnvesly and Naik (2001) pointed out the optimum pH and temperature requirement for maximum amount of protease production in chemically defined medium at pH 8-12 and 70 - 80 °C temperature. Santong *et al.* (2008) observed that 50 °C was the optimum temperature for maximum production of thermotolerent protease enzyme by raw milk isolate *Bacillus* sp. in LB broth with 2% skim milk powder.

The purification process of enzyme is important for the product recovery. There are various methods adopted for the purification of enzymes. An experiment showed that the protease produced by *Clostridium* sp. was purified by Sephadex G-100 column chromatography and ammonium sulphate precipitation methods (Singh *et al.*, 2006). Similarly, Siriporn *et al.* (2006) purified alkaline protease at three fold times by *B. megaterium* using butyl Toyopearl 650M, phenyl-Toyopearl 650 M column and Superdex 7510/300 GL column. El-Safey and Abdul-Raouf (2004) purified the protease in ammonium sulfate precipitation and Sephadex G200 filtration methods. In the present study, the protease produced by all the tested organisms was purified by two different methods *viz* Sodium alginate and Ammonium sulphate-butanol precipitation methods. Among these two methods, sodium alginate method showed maximum purification as well as comparatively more quantity of enzyme produced than ammonium sulphate butanol precipitation method.

Generally, molecular mass of the enzymes produced by the microbes are varied much. The thermostable protease by *Streptomyces tendae* was purified by Chi-Nam *et al.* (2004), they observed the molecular weight of this enzyme as 21 kDa. Similarly, Shin *et al.* (2004) reported that the molecular mass of the intracellular protease produced by *L. casi* was 55 kDa. Nakajima *et al.* (1974) were reported two extracellular proteinases produced by *Escherichia freundii* with the molecular weight of 51 and 41 kDa, respectively. Banerjee and Dutta (2006) pointed out that the protease recovery by acetone precipitation of mutant and parent *Pseudomonas* sp. observed by SDS-PAGE. It indicated that the purified protease from both strains migrated as a single band with 14.4 kDa in SDS PAGE, suggesting that the purified proteins were homogeneous. Similarly, an extra cellular protease from *P. flouresence* 22F was purified up to homogeneity by ammonium sulfate precipitation method. The purified enzyme had an apparent molecular mass of 52 kDa (Schokker and Van Boekal, 1997). Kazan *et al.* (2005) pointed out that the enzyme purified through DEAE cellulose chromatography from *B. clausii* GMBAE 42 by SDS-PAGE analysis revealed the molecular weight of 26.50 kDa. An organism, *B. proteolyticus* CFR 3001 was isolated from fish processing waste produced an alkaline protease; the molecular mass of this partially purified protease had 29 kDa. In the present study the protease produced by the test organisms grown on 1:2 ratio of milk substrate was purified and performed with SDS-PAGE analysis. From the results, it is understood that the molecular weight of the protease produced by the test organisms was ranged from 49.44 to 50.98 kDa.

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