RESEARCH PAPER | Microbiology | Volume : 4 | Issue : 12 | Dec 2014 | ISSN - 2249-555X

Purification of an Alkaline Protease Suited for Ecofriendly Sanitation in Milk Processing Units

KEYWORDS: alkaline protease, Bacillus cereus, sanitation

BEENA, A. K

Associate Professor, Dept. Of Dairy Microbiology, College of Dairy Science and Technology, Mannuthy, Thrissur, 680651

ABSTRACT *The objective of this work was to purify an alkaline protease suited for ecofriendly sanitation in milk processing units from bacteria of milk origin.Bacillus cereus isolated from sterilized skim milk was used for the production of protease enzyme.The crude protease obtained in this work performed maximally at pH 11.0. Enzyme retained 92.6 per cent of the original activity after 24 h of exposure to pH 12. Increased activity under alkaline conditions is suggestive of alkaline nature of enzymes.Qualitative assessment ofthe cleaning efficiency on proteinaceous soil confirmed the superiority of enzyme based formulation when compared to control.A purification fold of 141.31 was attained by ammonium sulphate fractionation, dialysis and column chromatography. SDS polyacrylamide gel electrophoresis under reducing conditions revealed a single band indicating that theenzyme preparation is homogenous.Based on Mf values, molecular weight of enzyme obtained in this study was approximated as 50.5 kDa.*

Introduction

Assurance of quality is an important mandate in the performance of food industry. Although food industry has undergone tremendous refinement in the field of food processing technology, the sanitation aspect which is of paramount importance has not received the deserved attention. As a part of quality assurance, major emphasis has so far been on 'hygiene' which in turn has eventually led to the indiscriminate use of detergents. The extensive use of chemical based detergents in the long run will adversely affect the ecosystem.The milk stones and biofilms, characterized by impervious protein matrix are identified as major hurdles that interfere with the efficiency of cleaning regimes in milk processing units. With greater thrust being given to environment protection, it is necessary to reduce the techno-genic contamination of natural resources. Enzymes, also known as 'green chemicals' are an ideal choice because of their biodegradability, low toxicity, non-corrosiveness and enhanced cleaning properties. It is conceived that microbial alkaline proteases having a high level of activity at a broad range of pH will be an ideal choice as cleansing additives in dairy industry. Identification of such enzymes and diligent use of these will be helpful in reducing the use of chemicals without compromising on quality. This could result in substantial savings in terms of water and energy, at the same time improving environmental performance. To identify enzymes specifically suited for dairy plant sanitation, an attempt was made to purify the alkaline protease produced by a bacterium of milk origin.

Materials and methods

Screening of milk samples for alkaline protease producers

Protease producers were isolated from milk(raw/pasteurized/sterilized) using skim milk agar as per the standard procedure of Harrigan, (1998).Those colonies that showed maximum zone of clearance after 48h were further assessed for their activity in alkaline pH bylooking into the zone of clearance after streaking on alkaline medium: Glucose Peptone Yeast extract Carbonate medium (Glucose-1%, Peptone 0.5%, Yeast extract- 0.1%, K_2HPO_4-0.1%, $MgSO_4$. 0.02%Agar – 1.8%)fortified with 10 per cent skim milk (Kumar, 1997).pH of the medium was adjusted to 10.0 using two per centSodium carbonate which was sterilized separately and added aseptically. Skim milk was sterilized separately and added aseptically to the medium at a level of 10 per cent (v/v) during plating.Those colonies that showed maximum zone of clearance in the alkaline medium were identified as thepotent alkalineprotease producer. Biochemical characterizationof the isolate was carried out (Barrow and Feltham,1995)

Production of protease enzyme and assessment of its pH stability

Standardized inoculum (OD at 600 nm – 0.6) of the isolatewas added at a level of three per cent to the optimized media with pH-11.0 and havingthe composition: Whey powder-, Skim milk powder,Tryptone,Trisodium citrate,Sucrose, and Magnesium sulphate each at alevel of 0.5%.After incubation at 37°C for 24 h, the media was subjected to refrigerated centrifugation (Eltek, RC 4100 D) at 8000 rpm for 40 min. The clear cell free supernatant(CFS) obtained was then filtered through 0.45µm Millipore filter. This filtrate containing the extracellular protease served as the crude protease enzyme.

The pH stability of the crude protease was assessed by exposing it to differentpH: 7.0, 8.0, 9.0, and 10.0 11.0 and 12.0 by mixing the enzyme with appropriate buffer(Tris-Hcl and glycine-NaOH buffer) in the ratio 1:1. The enzyme solutions at different pH values were incubated at 30°C for 24 h. The residual protease activity was subsequently determined in terms of tyrosine value, using 0.5 per cent casein as substrate. The original enzymatic activity before incubation at various pH values was taken as 100 per cent(Nilegaonkar*et al.*, 2007).

Assessment of cleaning efficiency of inbuilt formula

The cleaning efficiency of the inbuilt formulation containing crude protease was compared with that of a standard cleaning solution generally used in the laboratory, after artificially soiling the plates (Varghese, 2002). The cleaning efficiency of different combinations (Water alone, 0.5%Laboline; Tween 80:NaOH: Water:CFS (0.02:0.002:1:1);Tween 80:NaOH :water (0.02:0.002:2), were evaluated. The cell free supernatant (CFS) used in the trial possessed an activity of 25 U/ml.

Purification of protease

Fractional precipitation of extracellular enzyme was done

Volume : 4 | Issue : 12 | Dec 2014 | ISSN - 2249-555X

using Ammonium sulphate. The protein pellet obtained at 40 to 80% saturation was dissolved in minimum quantity of 20 mM TrisHCl buffer (pH 11.0) and dialysed using dialysis tubing 25mm x 16 mm (Sigma) against three changes of three litres of distilled water and then against the same buffer for 24 h.Precipitate formed was removed by centrifugation and the resultant solution was subjected to gel filtration with Seralose 4B (SRL).The fractions, containing protein was detected by measuring the absorbance at 280 nm using UV VIS Spectrophotometer (SL 159, Elico). Specific activity of all the fractions containing protein was determined by Lowry's method (Genei, Bangalore) using bovine serum albumin as the standard. Number of units of tyrosine per milligram of protein was taken asspecific activity of the enzyme. Fraction with maximum specific activity was again passed through Seralose 6B (SRL) and that fraction with maximum specific activity was rechromatographed with Seralose 6B, again. The fraction so obtained was than assessed for its homogenity by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) (Laemmli, 1970).

Results and discussion

Qualitative assessment of production of alkaline protease showed that maximum potential was exhibited by an isolate obtained from sterilized skim milk.Morphological and biochemical characteristics of the isolate were suggestive of the isolate to be *B.cereus*.Murphy *et al.* (1999) opined that *Bacillus* species with high proteolysis activities are so widely distributed in the environment that they could be introduced into milk and milk products at any stage of production and processing. Isolation of *Bacillus* species from sterilized milk is in agreement with Bellow *et al.* (2007) who have reported occasional occurrence of *Bacillus* originating from raw milk in high heat treated milks like UHT milk.

Zone of proteolysis

Assessment of enzyme activity on casein substrate after a preincubation at different pH for 24 h revealed that with increasing pH, enzyme showed a gradual increase in activity except at pH 12 where activity reduced by 7.4 per cent (table 1). A similar gradual increase in protease activity above pH 7.0 till pH 10 had been reported by Son and Kim (2002). Takami*et al.* (1990) reported that pH was important in designing the binding function between enzyme and substrate. Better activity at pH 11 might be due to the better binding of enzyme to the substrate at this pH.

Table 1.Stability of enzyme at different pH

pH of the medium	Protease activity (µmoles of tyrosine/ ml)	Residual activity (per cent)
7	314.34 ± 3.53	110.8[a]
8	325.41 ± 3.69	114. 3[a]
9	336.97 ± 6.54	118.7[b]
10	355.15 ± 2.06	125.1[c]
11	385.53 ± 3.13	135.8[d]
12	262.74 ± 2.59	92.6[e]

* The mean difference is significant ($p \leq 0.05$).

* Figures bearing same superscript do not differ significantly.

* Activity is expressed as µ moles of tyrosine released under standard assay conditions.

The present observation of maximum activity at pH 11 is in agreement with the reports of Joo*et al.* (2003) .Enzyme obtained in this work retained 92.6 per cent of the original activity after 24 h of exposure to pH 12. Stability of protease enzyme from *Bacillus* species over a pH ranging from 7- 12 had been reported earlier by Aftab*et al.* (2006). Such good stability at pH values up to 12 had been reported by Nogueira*et al.* (2006). This distinctive feature of high pH as optimum is a common feature of all alkaline proteases.

water wash

Plate 2a

Volume : 4 | Issue : 12 | Dec 2014 | ISSN - 2249-555X

Plate2 c

Inbuilt formulation with enzyme

Plate2 d

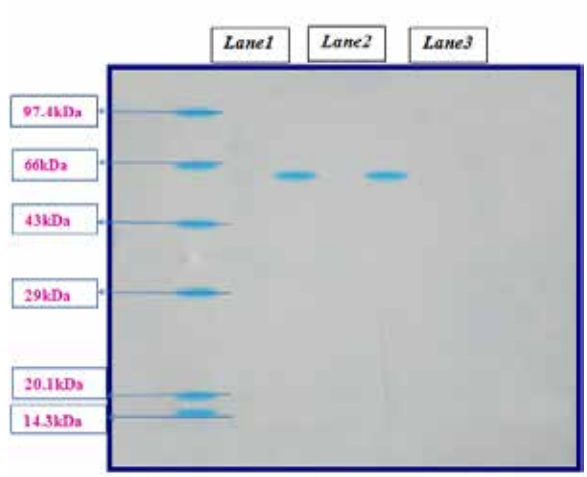
Inbuilt formulation without enzyme

Cleaning system followed in any process depends on the type of soil and nature of surface. According to Moore *et al.* (1951) soil accumulates on glass and stainless steel surface to a similar degree. In this study, soil was made artificially on glass Petri dishes using pasteurized whole milk as this was the most commonly encountered soil. According to Twomey (1975) protein contributes to more than 50 per cent of soil. Hence the crude protease enzyme obtained from isolate was assessed for its effect in improving the efficiency of cleaning formulations. When water alone was used, cleaning was not at all effective (Plate a). When labolene was used as per manufacturers' recommendations, the protein residues persisted, especially in the periphery, clearly indicating the lack of ability of cleaning solution to dislodge the dried soil (Plate b). When enzyme based formulation was used, obviously, dislodgement and clearing of plates was far better than the control (Plate c). Varghese (2002) also gave similar reports. To rule out the influence of detergent components other than crude enzyme, a cleaning formulation comprising of all components except enzyme was also checked for cleaning efficiency. Performance of this solution was very poor and the plates were characterized by a 'foggy' appearance, probably because of fat residue(Plate d). From the results, it can be concluded that crude enzyme did possess a complementary effect and that the inbuilt formulation comprising of cell free supernatant fared the best with regard to efficiency of cleaning.

Table 2. Enzyme activity at different steps of purification

Media	Tyrosine (µmoles/ml/min)	Protein (mg/ml)	Specific activity	Purification fold
CFS	92.6	0.121	765.00	1
40-80 per cent precipitate	412.08	0.022	18730.91	24.48
Seralose 4B	765.38	0.008	95672.50	125.06
Seralose 6B (27th fraction)	819.42	0.008	102428.20	133.89
Seralose 6B (39th fraction)	864.8	0.008	108100.00	141.31

(The removal of cells, solids and colloids from the fermentation broth is the primary step in recovery of extracellular enzymes. Inorganic sating out of proteins with ammonium sulphate was resorted to get 40-80 per cent fraction(Anustrup, 1980). The precipitate obtained at 40-80 per cent saturation was dissolved in minimum quantity of TrisHcl buffer (pH 11) and dialysed against the same buffer to get a purification of 24.48 fold.Dialysis and gel filtration has been used successfully for the purification of alkaline proteases by *Bacillus* species (Banik and Prakash, 2006). Of all the fractions collected,that fraction with maximum specific activity was passed through the column of Seralose 6B, The 39th fraction that showed maximum specific activity (102428.2) was rechromatographed through Seralose 6B to get fractions, wherein only 9th fraction showed presence of protein.The specific activity of this fraction was 108100 demonstrating that enzyme was purified by 141.31 fold. Vidyasagar*et al.* (2006) achieved a purification fold of 116 by gel permeation chromatography.



Lane1 – marker
Lane 2- purified enzyme
Lane 3- purified enzyme

Volume : 4 | Issue : 12 | Dec 2014 | ISSN - 2249-555X

The homogeneity of the purified protease (9thfraction after rechromatography throughSeralose 6B) from the isolate S4 was affirmed by SDS polyacrylamide gel electrophoresis under reducing conditions. The psychrotrophic spore former *B.cereus* was found to have the potential to produce alkaline protease that was compatible with surfactants.Ammonium sulphate precipitation; dialysis and gel filtration was effective in achieving a purification fold of 141.31.The work successfully purified and confirmedthe potential of protease enzyme to be used in dairy plant sanitation.

REFERENCE

1. Aftab, S., Ahmad, S., Saeed, S. and Rasool, S.A. 2006. Screening, isolation and characterisation of alkaline protease producing bacteria from soil. Pakist. J. Biol. Sci. 9: 2122-2126. | 2. Anustrup, K. 1980. Proteinases: Microbial Enzymes and Bioconversions.Economic Microbiology (ed. Rose, A.H.).Vol. 5.Academic Press, New York. 693p. | 3. Banik, R.H. and Prakash, M. 2006. Purification and characterization of laundry detergent compatible alkaline protease from B. cereus.Indian J. Biotechnol.5 (suppl.) 380-384. | 4. Barrow, C.J. and Feltham,R.K.A. 1993. Cowan and Steel's Manual for Identification of Medical Bacteria. Third edition. Cambridge University Press,London , 332p. | 5. Harrigan, W. F. (1998), Laboratory methods in food microbiology, (3rded). Academicpress, 242. | 6. Joo, H.S., Kumar, C.G., Park, G.C., Paik, S.R. and Chang, C.S. 2003. Oxidant and SDS-stable alkaline protease from Bacillus clausii I-52: Production and some properties. J. Appl. Microbiol. 95: 267-272. | 7. Kumar, C.G. 1997. Studies on microbial alkaline proteases for use in dairy detergents.PhD.Thesis, NDRI, Karnal, 248 p. | 8. Laemmli.V.K.1970.Clevage of structural proteins during assembly of head of bacteriophage T4. Nature 277: 680-685. | 9. Moore, O.R., Tracy, D.D. and Ordal, S.J. 1951. Permanent pipelines for dairy plants.J. Dairy Sci. 34: 804-814. | | | 10. Murphy, P.M., Lynch, d. and Kelly, P.M. 1999. Growth of thermophilic spore forming bacilli in milk during the manufacture of low heat powders. Int. J. Dairy Technol. 52: 45-50. | 11. Nilegaonkar, S.S., Zambare, V.P., Kanekar, P.P., Dhakephalkar P.K. and Sarnaik, S.S. 2007. Production and partial characterization of protease from Bacillus cereus MCMB-327.Bioresource Technol. 98: 1238-1245 | 12. Son, E.U. and Kim, J.I. 2002. Purification and characterization of caseinolytic extracellular protease from Bacillus amyloliquefaciens S94.J. Microbiol. 40: 26-32. | 13. Takami, H., Akiba, T. and Horikoshai, K. 1990. Characterisation of an alkaline protease from Bacillussp AH-101. Appl. Microbiol. Biotechnol. 34: 57-62. | 14. Varghese, S. 2002. Incorporation of mold enzymes to enhance the cleaning efficiency of dairy detergent formulations.M.Sc.Thesis, University of Agricultural Sciences, Bangalore, 123p p. |