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PARTIAL PURIFICATION AND CHARACTERIZATION OF ALKALOPHILIC PROTEASE FROM *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Partial purification and characterization of alkalophilic protease production from *Pseudomonas aeruginosa* was isolated from the gut of marine and coastal waters shrimp *Penaeus monodon*. The protease production was assayed in submerged fermentation to produce maximum protease activity (423 ± 0.09 U/ml). The enzyme was precipitated with ammonium sulphate and partially purified by ion exchange chromatography through DEAE Sephadex A-50 column. In 10^{th} fraction showed maximum protease activity (734 ± 0.18 U/ml) with increase in purification fold. The molecular weight of protease from *Pseudomonas aeruginosa* was recorded as 60 kDa. The stability of protease was tested at various pH and temperature; it showed maximum protease activity at pH-9 and temperature 50ºC. Among the various surfactants tested for enzyme stability, maximum activity was retained in poly ethylene glycol. The compatibility of protease enzyme with various commercial detergents; the enzyme retained maximum protease activity in tide. The results are indicated that all these properties make the bacterial proteases are most suitable for wide industrial applications.

Keywords: Alkalophilic protease; *Pseudomonas aeruginosa; Penaeus monodon*

INTRODUCTION

Microbial proteases are among the most important hydrolytic enzyme and have been studied extensively since the advent of enzymology. Proteases are necessary for life of living organisms being found in wide diversity, which performs both degradative and synthetic functions. A number of eukaryotic and prokaryotic organisms are reported to produce proteolytic enzymes. Marine microbes are reported to plays an important role in the earth system, being the major primary producers in the ocean and acting as a source of enzymes and antibiotics. The major microorganisms that are found in industrial protease producers exist in bacteria such as genera *Clostridium, Bacillus, Pseudomonas* and fungi like genera *Aspergillus, Mucor* (**Sakka *et al*., 1986**).

The extra cellular protease found numerous applications in industrial processes like in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes. Probably the largest application of protease in laundry detergents, where they help in removing protein based stains from clothing. For an enzyme to be used as a detergent additive, it should be stable and active in the presence of typical detergent ingredients, such as surfactants, builders, bleaching agents, bleach activators, filters, fabric softness and various other formulation aids (**Banerjee *et al*., 1999**).

In textile industry, proteases may also be used to remove the stiff fibre to achieve improved lustre and softness. Protease treatments can modify the surface of wool and silk fibres to provide new and unique finishes. Proteases have been used in the hide de-haring process, where de-haring is carried out at pH values between 8 and 10. An interesting application of alkaline protease was to decompose the gelatinous coating of x-ray films (**Ishikawa *et al*., 1993**). Proteases are also useful and important components in biopharmaceutical products such as contact lens enzyme cleaners and enzymatic debris (**Anwar and Saleemudin, 2000**). The preparation of chitin from various crustacean shells involves demineralization and deproteinization with the use of strong acids or bases. For alternative approaches to overcome the shortage and impacts of the chemical treatments, proteolytic microorganisms and proteolytic enzymes used for deproteinization of crustacean wastes has been applied (**Oh *et al*., 2000**).

Alkaline proteases are suitable for the detergent industry because of its stability at maximum pH (**Horikosi, 1996**). Proteases are currently received more attention in the view of their stability in high pH, temperature and in the presence of surfactants, organic solvents and denaturing agents. Proteases especially the alkaline proteases are physiologically and commercially important group of enzymes used primarily as detergent additives. Bacterial proteases are easily produced in large amounts, thermo stable and active at wider pH range. These properties make the bacterial proteases most suitable for wide industrial applications (**Banerjee *et al*., 1999**). The main objective of the study was the production and purification of protease enzyme from *P. aeruginosa*.

MATERIAL AND METHODS

Protease producing organism

The organism used in this study *P. aeruginosa* was isolated from the gut of *Penaeus monodon,* which was collected from the Rajakkamangalam marine coastal area in Kanyakumari District, Tamilnadu. Hence, an attempt was made to isolate and characterize protease producing bacteria from marine shrimp; the gut was dissected out from 4 or 5 shrimps in aseptic condition. Then one gram of gut sample were grounded well with sterile phosphate buffer saline (PBS) and serially diluted. Three consecutive dilutions were spread on the Zobell marine agar and colonies with different morphology were selected and purified in nutrient agar plates (**Kumar *et al*., 2011**). The bacterium was identified by following the standard key of Bergey's Manual of Determinative Bacteriology (**Holt *et al*., 1994**).

Effect of incubation period on enzyme production

The effect of incubation period on protease production was tested by *P. aeruginosa* inoculated on casein broth medium and incubated at 37°C. The culture growth was determined by read at 600 nm in UV-VIS Spectrometer and enzyme activity was estimated for every 6 hrs intervals until to reach a decline phase (**Kumar *et al*., 2002**).

Mass scale enzyme production

The protease producing organism *P. aeruginosa* was cultured in the production medium. The medium containing (g/l): casein-8g; glucose-10g; beef extract-5g; $CaCl_2$-0.1g; NaCl-30g; pH-9 in 1000 ml Erlenmeyer flask and inoculated with overnight culture (2% inoculums) of *P. aeruginosa* and incubated at 37°C for 72 hrs. The flask was kept in a shaker at 240 rpm. The cells were then harvested by centrifugation at 10,000 rpm for 15 min at 4°C and the supernatant was collected (**Banerjee and Ray, 2006**).

Ammonium sulphate precipitation

The enzyme was precipitated by addition of ammonium sulphate at a level of 60% saturation and the precipitate was stored at 4°C for until further analysis **(Jewell and Falkinham, 2000)**.

Purification of protease by Ion exchange chromatography

The protease enzyme was purified by Ion exchange chromatography through DEAE Sephadex A-50. The column was selected at a height and internal diameter of 30 cm and 1.2 cm respectively. It was gradually filled with the suspension of DEAE Sephadex A-50 and equilibrated with 20 mM Tris buffer (pH-8.2). After loading the sample (ammonium sulphate precipitate), the bound proteins were eluted with elution buffer (20 mM Tris buffer pH-8.2 + 0.5M NaCl). Fifteen, two ml fractions were collected at a flow rate of 6 ml^{-h} and protease activity, protein contents were analyzed in the each fraction **(Jewell and Falkinham, 2000)**.

Analytical methods

The protease activity was estimated as per the method of **Kunitz (1947)** using casein as the substrate. One unit of enzyme activity was defined as the amount of enzyme that released one microgram of tyrosine under standard assay conditions. The protein content was determined by method of **Lowry (1951)** using Bovine serum albumin (BSA) as the standard. SDS-PAGE was performed according to the method of **Laemmeli (1973)**. The Zymogram was performed by simple modification of the method followed by **Twining *et al.* (1993)**.

Characterization of protease

Effect of pH on protease activity

The effect of pH on protease activity was examined at various pH levels (pH 4-10). The optimum pH for protease activity was studied by the enzyme was pre-incubated with various pH buffers such as acetate buffer (4, 5), citrate phosphate buffer (pH 6), Tris-HCl buffer (pH 7-8) and carbonate buffer (pH 9-10) at 37°C for 30 min. The residual activity of protease was estimated under standard assay conditions **(Usharani and Muthuraj, 2010)**.

Effect of temperature on protease activity

The effect of temperature on protease activity was examined at various temperatures. The optimum temperature for protease activity was studied by the enzyme was pre-incubated with carbonate buffer (pH-10) at different temperatures ranging from 30 to 90°C for 30 min. The residual activity of protease was estimated under standard assay conditions **(Usharani and Muthuraj, 2010)**.

Effect of surfactants on protease activity

The effects of surfactants on protease activity were examined. The protease enzyme was pre-incubated with Triton X-100, Sodium dodecyl sulphate (SDS), Poly ethylene glycol (PEG), Tween-20 and Tween-80. The enzyme was incubated without any surfactant was taken as control. The residual activity of protease enzyme was estimated under standard assay conditions **(Doddapaneni *et al.*, 2007)**.

Compatibility of protease enzyme with various commercial detergents

The effects of detergents on protease activity were tested with various commercial detergents such as Ariel®, Tide®, Surf Excel® and Power®. The enzyme in the detergents was deactivated by heating at 100°C for 10 min. They were diluted in double distilled water to a final concentration of 7 mg/ml to stimulate washing conditions. The protease enzyme was pre-incubated with 5 ml of detergent solution at 50°C for 30 min. The enzyme was incubated without any detergent was taken as control. The residual activity of protease enzyme was estimated under standard assay conditions **(Devi *et al.*, 2008)**.

Statistical analysis

The parameters incubation period on enzyme production and pH, temperature and detergents on enzyme stability were statistically analyzed by one-way analysis of variance (ANOVA) using MS Excel to assess the effect of different variables **(Steel *et al.*, 1997)**. The value $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

The protease producing bacterium *P. aeruginosa* was isolated from the gut of *Penaeus monodon* were collected from the Rajakkamangalam marine coastal area in Kanyakumari District, Tamilnadu. Hence it was selected for mass production of protease enzyme, purification and characterization process. Most marine and estuarine organisms inhabit environment that are relatively rich in bacteria and other microorganisms. The estuarine and sea water may function as a medium for both transport and growth of microorganisms. Marine organisms, share on ecosystem with microorganisms which in turn responsible for their physiological activities **(Hansen and Olfsen, 1999)**. The gastro intestinal bacteria take part in the decomposition of nutrients and also provide the microorganisms with physiologically active material like enzymes, amino acids and vitamins **(Sugita *et al.*, 1997)**.

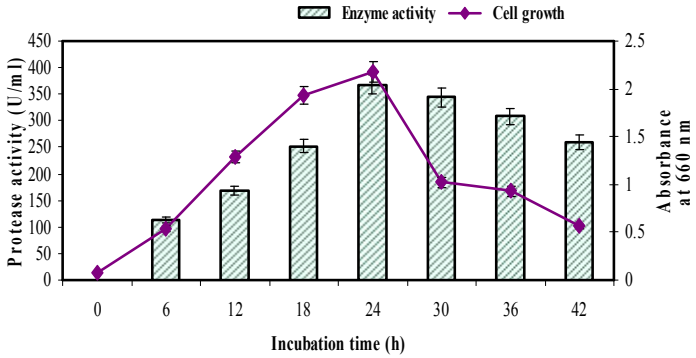

Figure 1 Effect of incubation period on protease production by *P. aeruginosa*

P. aeruginosa showed maximum protease activity (368 ± 0.25 U/ml) and higher growth rate at 24 h of incubation. It was significantly ($p<0.05$) **increased** the enzyme production and declined there after (Fig.1). Similar reports were reported by **Kumar *et al.* (2002)** protease production by *Pseudomonas* sp. S_{22} was found to be higher at 24 hrs of incubation period. The enzyme activity gradually decreased from 48 to 168 hrs. **Ferrero *et al.*, (1996)** stated that synthesis and secretion of the protease enzyme was initiated during the exponential growth phase and maximum protease production in the stationary growth phase.

Table 1 Summary of purification table for protease enzyme from *P. aeruginosa*

STEP	ENZYME ACTIVITY (U/ml)	PROTEIN (mg/ml)	SPECIFIC ACTIVITY (U/mg)	TOTAL ACTIVITY	PURIFICATION FOLD	YIELD (%)
Crude extract	423	2.5	169.20	21, 1500	1	100
Ammonium sulphate	506	1.2	421.66	16, 0230	2.5	75.76
Sephadex A-50	734	0.76	965.79	7, 3400	5.7	34.70

After mass scale production of protease enzyme, the cell free extract was obtained by centrifugation. It showed higher protease activity 423 ± 0.09 U/ml and specific activity was found to be 169.20 U/mg (Tab 1). The first step of purification of the enzyme was carried out by precipitation of protein from the cell free extract with ammonium sulphate at the saturation level of 60%. This resulted in 2.5 folds purification with yields of 75.76% and specific activity of 421.66 U/mg. The ammonium sulphate precipitate was applied to further purification process by using Ion exchange chromatography through DEAE Sephadex A-50 column, resulted in 5.7 folds of purification with yields 34.70% and specific activity of 965.79 U/mg. The higher protease activity (734 ± 0.18 U/ml) was produced at 10^{th} fraction when compared with other purified fractions (Figure 2). After purification process the specific activity was increased due to lack of contamination of other proteins.

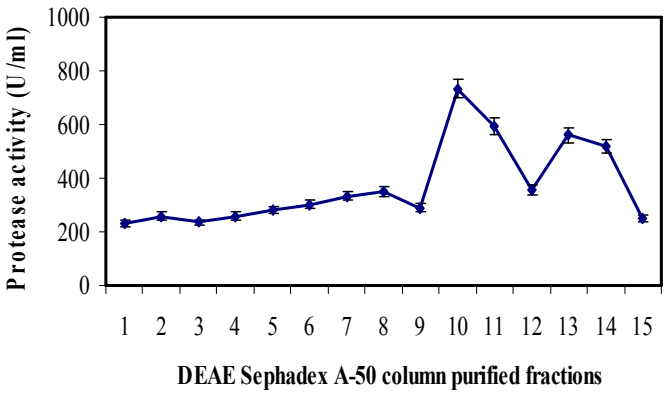

Figure 2 DEAE Sephadex A-50 column purified fractions of protease enzyme

The purified protease enzyme was recorded at a molecular weight of 60 kDa on SDS-PAGE. This band of hydrolysis at 60 kDa corresponded to a doublet band present in purified sample, due to an isozyme (Figure 3). The similar results were reported by **Jewell and Falkinham, (2000)** purified fractions of protease from *Burkholderia* strain exhibited a band of hydrolysis at a molecular weight of 60 kDa and doublet band was observed. Proteases from a number of different *Pseudomonas* species and one from *Burkholderia cepacia* have been purified and characterized. All of the proteases that have been purified to date are extra cellular metallo proteases isolated from culture supernatants. The molecular weight of these enzymes are found between approximately 20-60 KDa (**Sexton *et al.*, 1994**).

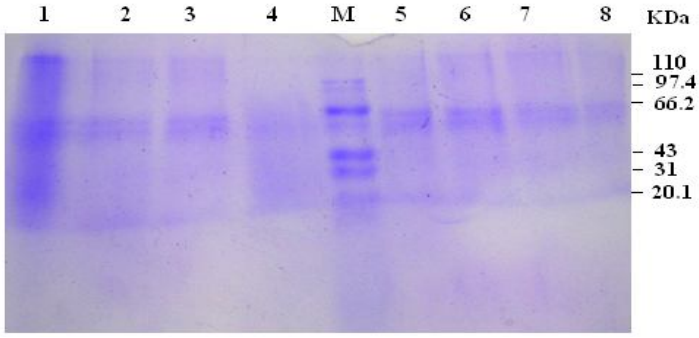

Figure 3 SDS-PAGE Profile of Protease enzyme (60 kDa)
Lane 1: Showing crude extract of protease,
Lane 2-8: DEAE Sephadex A-50 purified fractions of protease,
Lane M: Standard Protein Molecular Weight Marker.

After purification, the enzyme activity was confirmed by using Gelatin zymogram (Figure 4). Clear zone was observed on 10% SDS-PAGE gel with 0.1% of Gelatin as a substrate. Clear bands was observed on zymogram; indicate that this protease enzyme retained its activity in the presence of SDS and after complete washing with Triton X-100; resulted in restoration of protease activity. Similar results are reported by **Marquart *et al.* (2005)** a novel protease Las B obtained from *P. aeruginosa* and the identification of protease enzyme by gelatin zymogram after purification. The Las B protease enzyme retained its activity in treated with SDS and Triton X-100. Thus the enzyme was active in treating with SDS and Triton X-100 and due to this property it can be used as a detergent additive.

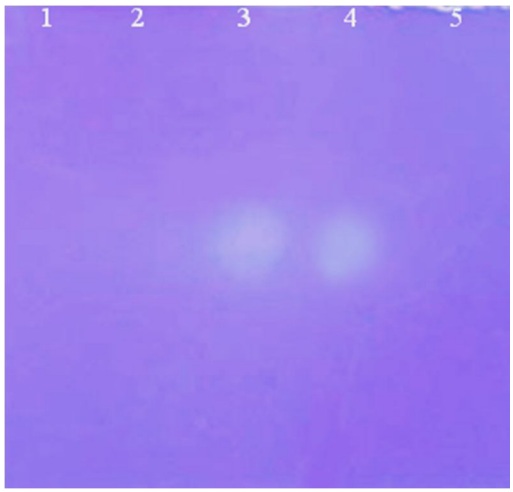

Figure 4 Zymogram analysis of DEAE Sephadex A-50 purified fractions of protease
Lane 3, 4: Showing purified fractions of protease hydrolyze the gelatin to form clear zone

The stability of enzyme was tested by pre-incubated with various pH buffers. Stability of enzyme was significantly higher at acetate buffer ($p<0.05$); the enzyme activity found to be 621.50 ± 0.11 U/ml. The stability of the enzyme was lowered at acetate buffer pH-4.0, the enzyme activity 30% decreased when compared to the optimum level (Tab 2). Similar results were reported by **Kanekar *et al.* (2002)** isolation and optimization of protease producing alkalophilic bacteria belonging to the genera *Bacillus, Pseudomonas* from sediment samples of the alkaline lake of India. The enzyme was stable at pH 8-12 and also active in the presence of commercial detergents.

Table 2 Effect of pH on protease activity

pH	RELATIVE ACTIVITY (%)
4	67.25
5	76.87
6	78.61
7	80.11
8	92.06
9	94.66
10	97.44

The purified enzyme was pre-incubated with carbonate buffer (pH-10) at various temperatures in the range of 40 to 90ºC. The stability of enzyme was significantly higher at 50ºC ($p<0.05$) and produces a maximum enzyme activity 645 ± 0.08 U/ml and the minimum enzyme activity (504 ± 0.17 U/ml) was observed at 40ºC. The enzyme retained its activity upto 90ºC, 30% of activity reduced from the optimum level (Tab 3). Similar results were reported by Sexton et al. (1994) the temperature range of activity of proteases of *Burkholderia* and *Pseudomonas* can range from as low at 4°C to as high at 60°C. **Srinivasan *et al.* (2009)** production of potential thermostable protease from *Bacillus* sp. isolated from tannery industry effluent. The activity of enzyme was stable upto 70˚C.

Table 3 Effect of temperature on protease activity

TEMPERATURE (ºC)	RELATIVE ACTIVITY (%)
30ºC	88.15
40ºC	89.17
50ºC	101.12
60ºC	86.83
70ºC	85.56
80ºC	81.47
90ºC	78.23

The effect of various concentration of SDS (0.1-0.5%) and Triton X-100 (1-5%) on protease activity was observed. In 0.2% of SDS showed maximum enzyme activity (543 ± 0.10 U/ml) and 0.4% of SDS showed minimum enzyme activity (283.8 ± 0.18 U/ml) (i.e.) 56% of activity reduced from the optimum level (Figure 5A). In 5% of Triton X-100 showed maximum activity (558.6 ± 0.12 U/ml) and 1% of Triton X-100 showed minimum activity (524.4 ± 0.29 U/ml), 18% of enzyme activity reduced from the optimum level (Figure 5B). Chu, (2007) also reported that the positive influence of Triton X-100 on protease activity by *Bacillus spp.* **Najafi *et al.* (2005)** reported that protease of *P. aeruginosa* PD 100 enable to act in the presence of SDS and Tween 80. The enzyme retained 50% of activity in 1% SDS and 3% Tween 80.

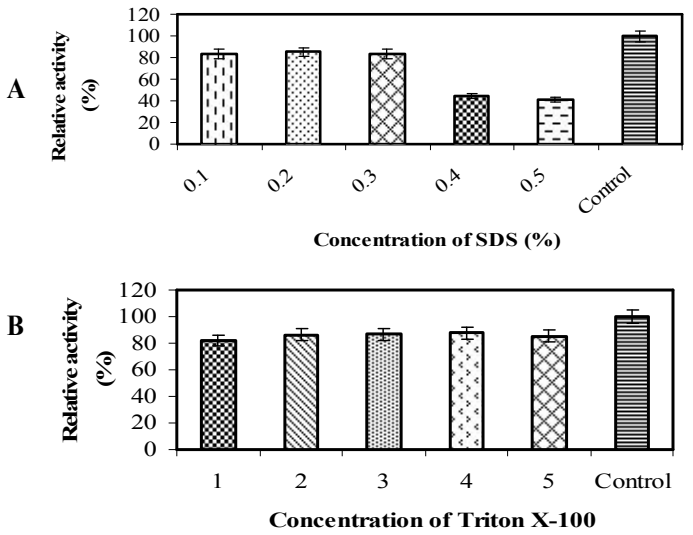

Figure 5(A) & (B) Effect of various concentrations of SDS and Triton X-100 on protease activity

The protease from *P. aeruginosa* was tested with various surfactants such as poly ethylene glycol (PEG), SDS, Triton X-100, Tween-20 and Tween-80. The enzyme retained maximum activity (637 ± 0.18 U/ml) at PEG and minimum activity in Triton X-100 (492 ± 0.12 U/ml). In SDS 87% of activity retained, whereas Tween-20 and Tween-80 showed, 84% of enzyme activity retained (Figure 6A). As the enzyme was stable with different surfactants it was confirmed as ideal enzyme to be incorporate in various detergent formulations. Similar results were reported by **Joo and Chang, (2005)** the protease from *Bacillus clausii* and *Bacillus* sp. which retained their activity with various surfactants such as Triton X-100, Tween-20 and SDS. **Beg and Gupta, (2003)** reported that the alkaline protease from *Bacillus megavensis* where 100% stability was observed for 1 hr at room temperature in the presence of various surfactants (SDS, T20, T40, T80, T85) and some oxidizing agents such as H_2O_2. The present attempt revealed that purified protease enzyme was pre-incubated with various detergents such as Tide®, Surf-Excel®, Power® and Ariel®. It was significantly ($p<0.05$) retained 95% of activity (666 ± 0.14 U/ml) in Tide® and only 74% activity (516 ± 0.12 U/ml) in Surf-Excel®. In Ariel® and Power® only 15% of activity was reduced from the optimum level (Figure 6B). Similar results were reported by **Devi *et al.* (2008)** alkaline protease producing strain *Aspergillus niger* isolated soil sample. Enzyme production was optimized under submerged conditions and it retained more than 50% activity after 60 min incubation at 40ºC in the presence of detergents such as Tide®, Surf-Excel®, Wheel® and Henko® indicating its suitability for application in detergent industry. The enzyme was stable and active with various detergents.

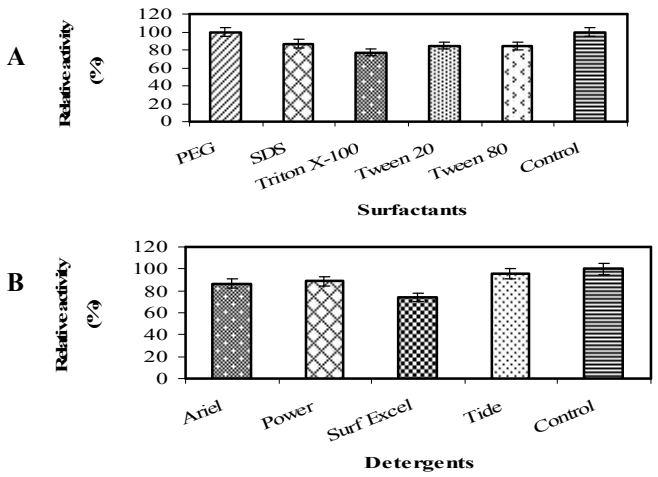

Figure 6(A) Effect of surfactants on protease activity was tested by enzyme pre-incubated with various surfactants. **(B)** Compatibility of protease enzyme with various commercial detergents.

The suitability of the protease for used as detergent additive is depended on its stability and compatibility with various surfactants at alkaline pH and wide range of temperature. The surfactants are perhaps the most important ingredients presented in every synthetic formulation **(Stoner, 2004)**. **Kanekar *et al.* (2002)** isolated and optimized the protease producing alkalophilic bacteria belonging to the genera *Bacillus, Pseudomonas*, from sediment samples of the alkaline lake in India. The enzyme was thermo stable (65˚C) at pH-12.0 and also active in the presence of commercial detergent. This enzyme removed blood stain from cotton fabric indicating its potential use in detergent formulations.

CONCLUSION

Proteases are currently received more attention in the view of their stability in high pH, temperature and in the presence of surfactants, organic solvents and denaturing agents. After purification steps the specific activity of protease enzyme was increased. The suitability of the protease enzyme for used as detergent additive is dependent on its stability in the presence of surfactants and compatibility with detergents at alkaline pH and in wide range of temperature.

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