LOW-LEVEL EXPRESSION OF ENZYME FROM MARINE BACILLUS SPECIES FOR DETERGENTS

ABSTRACT

Proteases are a varied and ancient family of enzymes that play a part in every aspect of an organism's functioning. As a member of the hydrolytic enzyme family, they help break down proteins into smaller peptides and amino acids. Proteases carry out a nucleophilic attack on the carbonyl carbon of the scissile bond to facilitate the addition of water. Because of the wide range of variances in their characteristics, such as substrate selectivity, active site composition, and catalytic mechanism, it is impossible to make generalizations regarding their stability and activity. Peptide synthesis, meat tenderization, cheese production, soy sauce production, silk industry organic syntheses, pharmaceutical industry waste treatment, protein hydrolyzation, and silver recovery from the waste photographic film are just some of the many applications of proteases, which have broad substrate specificity. Around 60% of all enzymes sold in the world are proteases derived from plants, animals, and microorganisms.

Keywords: Enzymes, Cellulose, APR-4 protease, Bacillus sp. APR-4

1.1 INTRODUCTION

It is possible for extremophile bacteria, which flourish in conditions that other creatures are unable to tolerate, to thrive. Since the early 1990s, thanks to Horikoshi and colleagues' pioneering work, the enormous potential of alkaliphiles (syn. alkalophiles) has been realized among these creatures. Alkaliphile-derived products have been commercialised successfully in the detergent and food industries. The industrial output of alkaliphile products is currently insufficient to meet demand. According to a study, it is estimated that the global enzyme market is worth $5.1 billion and that demand will increase by 6.3 percent per year. Production of ethanol and animal feed is predicted to increase the need for process-specific enzymes. Despite the fact that industrialised countries are more likely to increase their market

share, the analysis predicts that emerging countries will increase their market share the most. Alkaline enzymes make up a large portion of the enzyme market as detergent additives. As a result, alkaliphiles, the organisms that produce the great majority of commercial enzymes, need to be studied more closely. For example, alkaliphilic bacteria are capable of producing commercially available enzymes and other intriguing chemicals. Alkaliphile enzymes are detergent-stable because they tolerate high pH. For commercial detergents, these enzymes are also tested for their ability to survive in the presence of bleach. This means that washing with enzyme-based detergents uses less water and energy since they are more efficient because they may be used at a lower temperature. Alkaliphilic bacteria have also been found in acidic soil, in addition to those found in environments with a pH of 7 or above. It is more likely that alkaliphiles will flourish in acidic or neutral environments because of the presence of alkaline pockets. Alkaliphiles are creatures that can either be required or optional depending on the situation.

There are several types, including meso-, thermo-, psychro-, and haloalkaliphiles. To develop to their full capacity, most true alkaliphiles prefer soils with a pH of at least 9.0 and preferably 10.0. Alkaline conditions are those that have a high or low calcium concentration. For the creation of biomolecules appropriate for industrial usage, haloalkaliphiles, and thermoalkaliphiles are promising. This group of microbes' enzymes has been used in a wide range of industries, from textile finishing to pulp and paper production to food processing and even laundry detergents.

1.2 COMPOSITES

Proteases are among the most significant industrial enzymes. Although alkaline proteases make up just 25 percent of the enzyme market, their sales make up 60 percent of the global market share. Bacillus serine protease secretion by Bacillus spp. strain 221 was first found by Horikoshi and has subsequently been studied, described, and commercialised multiple times. Contact lens solutions, detergents, meat and cheese processing, and silver recovery from photographic films all require proteases. These enzymes have also helped to limit the quantity of toxic waste that is generated during the manufacturing process. Enzymatic silver recovery procedures, for example, are less hazardous to the environment than more conventional ones like burning films (which emit harmful gases). In 1913, Burnus became the first enzyme detergent to be

composed of pancreatic trypsin and sodium carbonate extract, while BIO-40 was launched in 1956. Proteases must be able to function well in alkaline pH and high temperatures to be used in detergents. A further advantage of broad pH and temperature range proteases is that they can withstand more rigorous industrial production processes. Surfactants and other detergent composition additives should not impair the activity of the protease. In order to be an efficient detergent ingredient, the pH of the detergent formulation must match the protease pI. Serine proteases derived from bacillus members have commercially used detergent proteases. Commercial success has been achieved by alkaline serine proteases, of which subtilisins are prototypes. These proteases, which are active in the pH range of 7.0–11.0, are essential components of laundry detergents. Bacillus is responsible for the production of all of the main subtilisins. Alkaline proteases have been widely studied in the industrial setting. In 2002, Gupta's group published a comprehensive analysis of commercially available alkaline proteases that have been successfully employed in detergent formulations, silk degumming, the food and feed sector, photographic gelatin hydrolysis, leather dehairing, cosmetics, and medicines. Alkaline proteases have recently shown features that make industrial scale-up feasible. There have been new reports of isolated proteases that not only function at low temperatures but also tolerate salt and organic solvents, indicating that they could be used in industrial settings. It was cloned from *Pseudomonas aeruginosa* strain K and the alkaline protease gene was amplified and characterised. The activity of purified protease was found to be 1.0112 U/m

1.2.1 Proteases derived from microorganisms

Proteases from microorganisms are often used in biotechnological applications because they have many of the qualities and characteristics needed. It is true that microbes may release large amounts of enzymes utilizing low-cost substrates, and DNA or RNA can be easily manipulated to create a huge amount of a desired enzyme. A simple and easy way to make enzymes extracellularly is to use the product of interest. It is also possible to keep the enzymes for several weeks without a substantial loss of activity with microbial proteases, which have a longer shelf life.

1.2.2 Serine protease enzymes

In the active site of a serine protease, a serine group is present. Each of the 20 families has been further divided into around six clans based on their shared ancestors and their structural similarity. In addition, serine proteases have glycine residues near the catalytic serine residue to form the motif Gly-Xaa–Ser–Yaa–Gly. This is an interesting trait. Through their irreversible suppression by PMSF, DFP, 3, 4-DCI, E.64, and TLCK, they are recognised as the inhibitors of a wide range of cellular processes, including apoptosis and apoptotic cell death (Tosyl-L-lysine chloromethyl ketone). pCMB (p-chloromercuribenzoate) inhibits several enzymes because it contains a cysteine residue near the active site. The optimal pH range for serine proteases is between 7 and 11, with neutral or alkaline being the most active. Esterolytic and amidase activities are just two examples of their diverse substrate specificities. Amounts between 18 and 35 kDa are typical for these molecules. pH 4 to pH 6 is a common range for their isoelectric points. Proteases like trypsin and chymotrypsin have been extensively investigated in this group. Most serine proteases are serine alkaline proteases that are active at high alkaline pH.

1.2.3 Proteases of Aspartic Acid

This enzyme is usually referred to as an acid protease. The catalytic activity of these proteases is dependent on aspartic acid residues. Clan AA includes pepsins (A1), retropepsin (A2), and enzymes from pararetroviruses (A3), all of which have been classified into three families. The isoelectric points of most aspartic proteases are in the pH range of 3 to 4.5 and show maximal activity at pH 3 to 4 (pH 3 to 4). In the 30 to 45 kDa range, you'll find their atoms and molecules. The active-site cleft is positioned between the two lobes of the pepsin family. The pattern Asp-Xaa-Gly contains the active-site aspartic acid residue, where Xaa might be Ser or Thr. In the presence of copper ions, pepstatin and diazocompounds such as EPNP [1,2-Epoxy-3-(p-nitrophenoxy) propane] inhibit aspartic acid proteases and DAN (Diazoacetyl-DL-norleucine methyl ester).

1.3 AMYLASE

The majority of our meals consist of starch, which can be found in leftovers, utensils, and clothing. Biocatalysis of starch-based products has found extensive use in amylases. Biocatalytic hydrolysis of glucosidic linkages in starch is mediated by a number of enzymes: a-amylase (1,4-a D-glucan glucanohydrolase; EC 3.2.1.1), glucoamylase (1,4-a-D-glucan glucohydrolase; EC 3.2.1.3), b-amylase (1,4-a-D-glucan maltohydrolase; EC 3.2.1.2), and a-glucosidase. 1,4-glucoside deglucosidase enzyme (EC 3.2.1.21) Debranching enzymes include isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41) (glycogen a-1,6-glucanohydrolase). Glycose, malt, and maltotriose are formed as a result of the action of a-1,4 bonds between adjacent glucose units. About a quarter of the industrial enzyme market is made up of extracellular enzymes, which are mostly employed in the food industry for operations like brewing, baking, and fruit juice processing. Breaking down the second a-1,4 glycosidic link results in maltose units being formed. In contrast to the slower action of b-amylases, the substrate can be arbitrarily cleaved by a-amylases. Amylase and amylopectin are broken by glucoamylases, which cleave the a-1,6 glycosidic connections and the final a-1,4 glycosidic links, releasing glucose units from their non-reducing ends. Because they prefer an acidic pH, they are not compatible with the b-and a-amylases.

1.4 CELLULASE

D-glucose units are linked by b-1,4-glucosidic linkages to form cellulose, an important part of plant cell walls. The use of cellulose-based substrates as sustainable sources of bioenergy is being studied around the world. Cellulose is readily available in agricultural wastes, but the high cost of conversion prevents it from being fully utilised. b-1,4-glycosidic linkages are hydrolyzed to release glucose units by cellulase, an enzyme In order to improve the conversion of cellulose-containing wastes, this enzyme is essential. Cellulases are found in many organisms, including fungi, bacteria, flora, protists, and insects. To produce glucose, 3 types of cellulases work together to break down cellulose sequentially. Cellobiose is hydrolyzed by exo-b-1,4-glucanases, endo-1,4-b-D glucanases, b-1,6-glucosidases, and endo-1,4-b-D glucanases at random, resulting in glucose from the hydrolysis of cellobiose. In some

cases, cellulases are able to perform both endo and exo functions. Exoglucanases and endoglucanases work together to solubilize the cellulose crystal.

1.5 ENZYMES FOR INTIMATE CARE AND EMERGENCY RESPONSE

Modern household detergents are the greatest application field for enzymes nowadays. Enzymes have contributed substantially to industrial detergents' development and improvement. The mild temperatures and pH levels of modern laundering conditions make them successful, and they contribute to Laundry, dishwashing, and institutional and industrial cleaning.

- Overall cleaning presentation has been boosted.
- Cellulases operate on cotton fibres to rejuvenate the cloth.
- Reduced energy usage can be achieved by lowering the temperature of the washer.
- Because the soil is released more efficiently, less water is used.
- Biodegradability means they have a little environmental impact.
- Improved water effluents for the environment (particularly those that are phosphate-free and less alkaline)

1.5.1 Washing-machine Enzymes

Water-soluble detergent enzymes are the most commonly employed to remove soil containing proteins, fatty acids, and carbohydrates. Efforts are now being made to increase the number of enzymes that can be used in detergent. There are a lot of problems with modern food, like ice cream for babies and sweets, dressings, and sauces. These and other conventional soilings, such as grass, eggs, blood, and animal and vegetable fat, can be removed from detergents by adding a variety of hydrolases. Lipases, proteases, cellulases, mannanases, amylases, and pectinases are the most common classes. Because proteases are used so often in laundry detergents, they were the first of their kind to be used a lot. When cotton-based textiles are used cellulases can help keep their appearance or even make them look new again through certain reactions that cellulases couldn't do before. Lipases are an important part of enzyme solutions that can be used to replace surfactants because they can work as an

alternative to current surfactant technology when it comes to removing greasy lipid-based stains.

2. LITERATURE REVIEW

Paula Monteiro (2010) Amylases are one of the most commonly utilised enzymes in business. To break down starch into glucose-based polymers, these enzymes use hydrolysis. Animal, plant, and microbial sources of amylases can be used in a wide range of commercial activities, including the pharmaceutical and food processing sectors. Enzymes derived from fungi and bacteria, on the other hand, have dominated industrial applications to date. To convert starches into oligosaccharides, alpha-amylase must be synthesised. Several commercially significant crops, such as cassava, maize, potatoes, maize, and rice, use starch as a crucial storage product. They make maltodextrin, modified polysaccharides, sugar and fructose syrups, and more. Starch-converting enzymes make them all. Various microbial "alpha-amylases" are used in the detergent, food, paper, and textile industries. When alpha-amylases were made in the past, they were made through submerged fermentation. Solid-state fermentation systems could change how this was done in the future. All of the alpha-pH amylases are important to the development of the fermentation process because of their stability, higher stability, pH profile, and independence from Ca?? or Calcium ion??. Since the enzymes' chemical, physical, and structural properties, as well as their industrial uses, make up the bulk of this review, alpha-amylases are the primary focus.

Adrio and Theodore (2014) are Microbial enzymes play a critical role in the development of industrial bioprocesses. Medicine, beverages, food, and textiles are just a handful of industries that have already reaped the benefits of this technology. New, upgraded, or more flexible enzymes now need to be used in order to come up with new production methods that are more innovative, long-lasting, and cost-effective now. Genomic and metagenomic methods are being used by researchers to discover new microbial enzymes that can be enhanced or modified using a wide range of methodologies that are based on rational, semi-rational, or random-directed evolution. Most commercial enzymes are made by bacteria and fungi, both of which can be found in the wild.

Ms. Mala, (2010), An alkaline protease-producing Bacillus strain isolated from laundry soil was used to investigate detergent biocompatibility. It was determined that the morphology and the nucleotide sequence of the 16S rDNA of Bacillus species Y were closely related to *Bacillus cohnii* YN-2000. Some 50–70 percent saturation of ammonium sulphate did wonders for the purity of the enzyme. SDS-PAGE was able to show the difference in a half-isolated enzyme. and casein zymography revealed two isozymes with molecular weights of 18 kDa and 66 kDa, respectively. The enzyme was most active when the pH was set to 12 and the temperature was set to 50 degrees. At pH 12, 60% of the enzyme's activity could be sustained for a 5-hour period of time. This is great news: The 44 percent of the enzyme's activity that was kept going for up to two hours at 50°C was very good hydrolysis selectivity demonstrated by the protease on a wide range of substrates. In the presence of Co2+, Mn2+, and EDTA, protease activity was significantly elevated. Bacillus Y can be used in detergents because three local detergents (Aerial Tide, Kite) were found to be compatible with the enzyme at 30°C and 50°C for up to three hours. This shows that this enzyme could be used in detergent applications.

The Guptas (2002) Proteolytic enzymes are essential for cell proliferation and growth in all living organisms. Extracellular proteases have a wide range of commercially important industrial applications. Although many microbes can produce proteases, only a few of these organisms have been found to be economically viable. Bacteria-based alkaline proteases like savinase (also known as subtilisin Carlsberg) and subtilisin BPN′ are often found in dishwashing detergents. Proteases may have become more stable through changes in wash conditions, heat, and oxidising agents. Site-directed mutagenesis and/or random mutagenesis have been used to generate novel preparations such as Maxapem, Durazym, and Purafect. Specific and stable subtilisin variants have been created through directed evolution as well. The development of molecular imprinting using conditional lyophilization for protein engineering is now underway. Biocatalysts can be modified using a wide range of molecular approaches. It is hoped that the "metagenome" method of finding more natural sources of alkaline protease will show a level of molecular diversity that has never been seen before. This is a big deal because it opens the door to the biotechnological use of microorganisms that had never been grown before. This is a huge resource that currently outnumbers the species that can be grown.This review

talks about ways to increase the amount of proteases that can be made, new ways to make new proteases, and how alkaline proteases can be used in industrial areas. The detergent industry is the company's primary emphasis.

Shallmey and Marcus (2004) found that for microbial fermentations, Bacillus species continue to be the dominant bacteria. Bacillus subtilis (natto) is the principal microbe involved in conventional soybean natto fermentation. The FDA's GRAS (generally recognised as safe) list includes a number of Bacillus species. Several Bacillus strains are among the most important industrial producers of these enzymes due to their ability to release substantial volumes (20–25 g/L) of extracellular enzymes. It's because different species of the genus can ferment at different pH levels, as well as because there are also thermophiles in the genus, that a lot of unique commercial enzyme products have been made. Conventional mutation and selection methods were used to make these products, but new cloning and protein engineering techniques were also used to make them. For a long time, it was believed that Bacillus proteases prevented Bacillus hosts from producing and secreting large amounts of foreign recombinant proteins. Gram-positive bacteria are more vulnerable to wall-associated proteases because the folding of heterologous proteins at the membrane–cell wall interface takes longer than it should. Disulfide-bound proteins may also benefit from the presence of enzymes that convert disulfide atoms into thiols, This may help *B. subtilis* to sectere enzymes. New insights into the complex protein translocation process of gram-positive bacteria could make Bacillus species the most important hosts for heterologous protein production if we can solve present secretion issues. Bacillus strains that have been industrially modified produce nucleotides, the flavouring agent ribose, the vitamin riboflavin, and poly—glutamic acid. Bacillus species, such as *B. subtilis* 168 have become a favourite host for many new and better products as we move through the genomics and proteomic eras.

Almeida do Nascimento (2006) Detergents, medications, and food all rely on protease enzymes, which have become increasingly important in industrial processes. Bacillus is the primary source of proteolytic enzymes found in Ariel, Tide, and Biz detergents. In this study, thermophilic Bacillus sp protease compatibility with commercial laundry detergent is examined. There was still a lot of this enzyme's activity after one hour at 60 ^{o}C. $CaCl_2$, $MnSO_4$, and Glycine kept 95% and 74% of their peak activity,

respectively. At concentrations of 1.0-5.0 mM, EDTA had no inhibitory effect. There was no dose of Triton X-100 that did not inhibit the enzyme in some way. The enzyme became unstable in a 5 percent (v/v) peroxide solution. After 30 minutes of incubation at 60oC with Tide® and Cheer® detergents, more than 80% and 66% of the protease's activity was still there.$CaCl_2$ and glycine helped the enzyme in Tide® detergent keep more than 86% of its activity after one hour of incubation.Proteases from Bacillus species can be used in laundry detergents based on these findings.

3. METHODOLOGY

3.1 Isolation of bacterial isolates

A total of four locations in Rajshahi and Sylhet, Bangladesh, were surveyed for their bacterial composition. Throughout the months of April and May of 2016, these included silt from the Padma River, hill soil from Sylhet, rhizosphere soil from bean roots in Saheb Bazaar, and sediment from Chalan Beel. We used a sterile inoculating loop/spoon to collect samples from at least 10 cm depth in each location. A pre-sterilized Eppendorf was used to transport the sample to the USDA-lab at Shahjalal University of Science and Technology. The samples were then dried in an oven at 70 degrees Celsius for an hour.

3.2-Nodulation of Seeds and Strains of Bacteria

APR-4, a Bacillus sp. strain, was earlier discovered here at 50°C. To make the seed culture, it was cultivated for 24 hours at 50°C and 165 rev/hr in an incubator shaker on a medium containing glucose-10, NaCl-5, peptone, yeast extract, $MgSO_4.7H_2O$, and $CaCl_2.2H_2O$, pH 7.0.

3.3. Enzyme Production

A seed culture (5% v/v) was added to the medium and agitated at 165 rev/min for 40 hours at 50 °C. The culture was centrifuged at 10,000 g for 35 minutes at 5°C. The supernatant was used to extract a crude enzyme.

3.4 Assays for the Detection of Proteases

The enzyme was shown to be active. 4 ml of casein solution (2.5 percent w/v in 50 mM Tris HCl, pH 9.0) and 1 ml of the enzyme were used in the assay combination. acid (5%) was added after 5 minutes of incubation at 65°C and vortexed for 5 minutes after chilling on ice. After filtering through Whatman 1, the optical density of this mixture was compared to the control at 275 nm. The amount of enzyme needed to break down one gram of tyrosine per minute was called a unit of enzyme activity (U), and that was how many units of enzyme activity were used.

3.5 Bacillus sp. APR-4 protease stability in detergents and surfactants

Proteinase was incubated for 30 minutes at 50°C with 5% (v/v) detergents (Wheel®, Surf, Tween-80, Tween-20, and Triton X-100). After 60 and 30 minutes of incubation at 65°C, residual activity was evaluated against enzyme control. A range of concentrations of both Farishta® and Fena® were used to test the enzyme's stability for 90 minutes at 30 degrees Celsius in a range of concentrations, from 3 to 20% v/v, which is equal to 15 mg/ml. Then, for 24 hours at 30 degrees Celsius, the enzyme was kept at the same concentration for that time.

3.6 Effects of Bleaching and Oxidizing Agents on the Environment

To find out how much of the enzyme's activity remained after it had been incubated for an hour at 50°C with different concentrations of sodium hypochlorite and hydrogen peroxide, we looked at the enzyme's residual activity.

3.7 APR-4 Protease Wash Performance of Bacillus sp.

Temperatures of 40, 50, and 65 °C were used in this experiment, with Erlenmeyer flasks that contained 300 cc of egg yolk-stained cotton

- only water
- Making use of water and washing soda (2%, 20 mg/ml)
- In water, the detergent (2 percent, 20 mg/ml) and the enzyme (100 U/ml) in water are added. Also, at 70°C, the same treatment was applied to the blood-

stained test fabric. The effectiveness of stain removal was evaluated through the use of visualisation.

4. RESULTS AND DISCUSSIONS

Table 1. Stability of protease of Bacillus sp. APR-4

Detergents /Surfactants (5 % v/v of 10 mg/ml)	Relative activity (%)	
	After 30 min	After 60 min
Wheel	98	98
Fena	107	100
Farishta	112	107
Surf	8	7
Tween -20	90	90
Tween -80	92	92
Triton X- 100	92	93

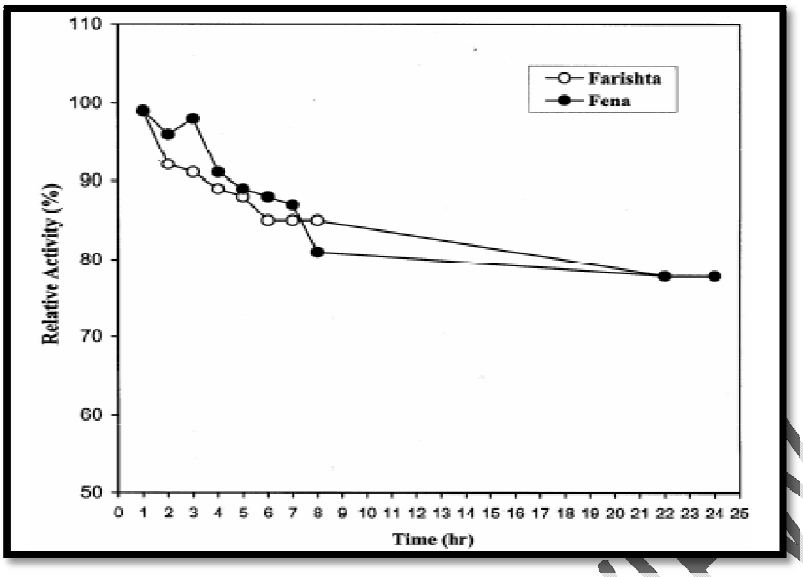

Fig 1. After incubation APR-4 protease of Bacillus sp

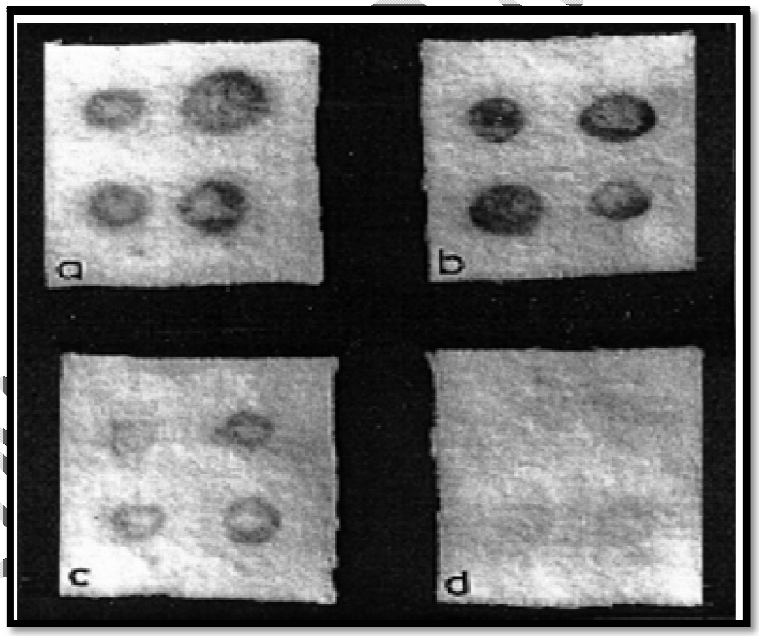

Fig 2. Protease from Bacillus sp. APR-4 on egg yolk staining

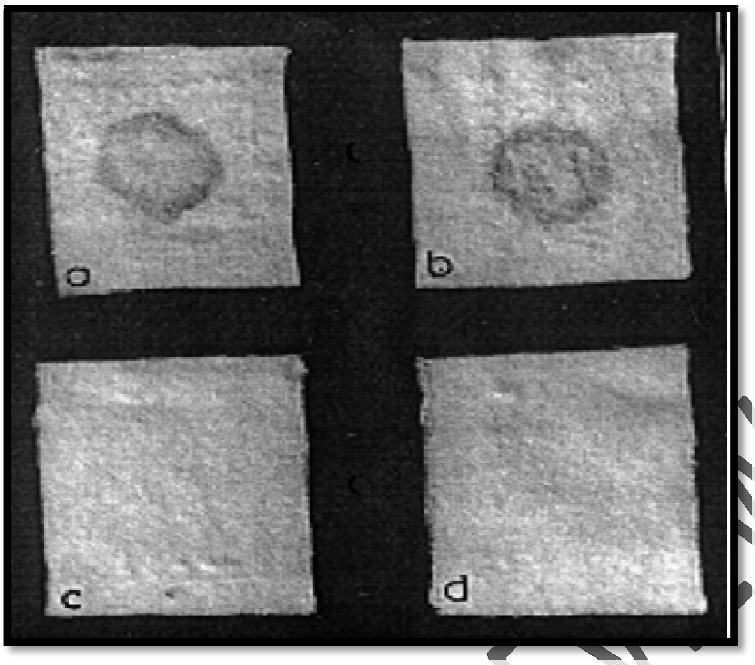

Fig 3. Effects of APR-4 on bloodstain

Bacillus sp. APR-4 is most active at a temperature of 65℃ and a pH of 9. The half-life at 70 and 80℃ was 150 and 90 minutes, respectively, with a half-life of 24 hours at 40℃. After 30 minutes of incubation, Farishta and Fena increased *Bacillus* sp. APR-4's enzyme activity. After one hour of incubation at 50°C, Fena did not stimulate activity, but Farishta had an 8% higher activity than the control. With Wheel present, the enzyme preserved 98% of its residual activity, while Surf reduced it to 7% after one hour of incubation. In the presence of non-ionic detergents (Tween-20, Triton X-100Tween-80), enzyme activity was reduced somewhat after one hour of incubation at 50°C. In general, enzyme stability is unaffected by non-ionic detergents, but the cause of the decrease in enzyme activity in this study is unknown. Enzyme stability was tested at various concentrations of detergents (Farishta, Fena) after 90 minutes of incubation at 30℃. The enzyme was relatively stable in Farishta and Fena at 3% (v/v) and 6% (v/v). The residual activity in Farishta (38%) and Fena (7%) diminished when the detergent concentration was raised to 20% (v/v), and this may be owing to denaturation of the enzyme occurring at higher detergent concentrations. Egg yolk stains were removed from cotton fabric using 100 U/ml of Bacillus sp. APR-4 protease and 1 percent (v/v) detergent at 40, 55, and 65 degrees Celsius (Farishta).

Egg yolk stains could be eliminated in 1 hour and 30 minutes at 55°C but required 2 hours and 30 minutes to remove at 40°C. When enzymes were not available, stain removal took a long time. The stain was removed in 10 minutes at 65°C with 100 U/ml of enzyme and 1 percent (v/v of 15 mg/ml) of detergent (Farishta) after water was replaced with buffer (50 mM Tris HCl, pH 9.0). After 30 minutes at 65°C with enzyme (100 U/ml) alone, the enzyme totally removed bloodstains from the test cloth (cotton).

5. CONCLUSION

Historically, many alkaline proteases have been used as biobuilders in industrial laundry detergents to hydrolyze and remove proteinaceous components from soiled garments. Several experiments using both regular clothing and conventional test patches stained with proteins demonstrate the efficacy of protease in eliminating proteinaceous stains, despite differences in washing conditions and detergent formulations. Bacillus sp. APR-4 protease was useful for synthesising peptides and removing gelatin from used X-ray films. Bacillus sp. APR-4 shows promise as an enzyme for laundry additive/detergent formulations due to its stability in detergents and hypochlorite and its ability to improve wash performance.

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