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RESEARCH ARTICLE

PURIFICATION AND CHARACTERIZATION OF BLOOD STAIN DECOLORIZING ALKALINE METALLOPROTEASE FROM BACILLUS SUBTILIS IAS 01 FOR PROMISING BIO-DETERGENT

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ABSTRACT

Alkaline Protease from *Bacillus subtilis* IAS 01 (KF:761633) was successively purified by 70% ammonium sulfate precipitation, ion exchange chromatography on DEAE-cellulose and gel filtration chromatography on Sephadex G-100 with 10.45 % recovery and 11.63 fold increase in specific activity. The molecular weight of the purified protease was determined as 14.3 kDa under SDS-PAGE, respectively. The characteristic of 70% $(NH4)_2SO_4$ precipitated protease was active at 55°C and pH 12.0. Among the tested mono Na^+ (76.42±1.41%), divalent ion $CaCl_2$ (130.88±1.48%) represent the highest activity and highly stability towards non-ionic, ionic surfactants, commercial detergent and 1mM EDTA inhibited by 94.2% of its original activity indicated alkaline type of metalloprotease. In addition wash performance was exhibited that the 70% ammonium sulfate precipitate metalloprotease could successfully remove the dried blood stain from hospitalized bet cloth within 30 min than commercial detergents. Considering its promising properties, *Bacillus subtilis* IAS 01 enzymatic preparation may be considered a potential nominee for bio-detergent in future use, respectively.

INTRODUCTION

Recently, microbial proteases are used for alternative method of detergents formulation and variety of industrial application in all over the country around 60% (Banik *et al*., 2004). Proteases also help to prevent the re-deposition of proteins on fabrics, particularly hydrophobic ones present in soils, such as blood, thereby also providing a whiteness benefit (Nasri *et al*., 2009). The continual exploration of enzymes and their utilities have expanded their industrial usage with the growth of 7.6 % per annual reported by (David *et al*., 2009). In this context, alkaline proteases of microbial origin have been extensively studied however detergent stable proteases were characterized from a few bacterial species (Deng *et al*., 2010). Generally, protease are protein hydrolyzing enzyme that cleavage the peptide bond between amino acid sequences and they are divide into acid, neutral and alkaline protease based on the optimum activity of the pH and also classified into serine protease, cysteine protease, metalloprotease and aspartic protease constitute one of the important groups industrial enzyme around the world (Masse *et al*., 1983; Barrett *et al*., 1998). In addition to the thermo-alkaline stability, minimum loss of enzyme functions in the presence of chelating agents (such as EDTA) is another invaluable property of industrial enzymes, particularly proteases (Asoodeh *et al*., 2012). To read the cleaning performance of alkaline proteases in detergents depends on number of factors including the pH of detergent, ionic strength, temperature, detergents composition and washing procedure. However, the key challenge for the use of enzymes in detergents and it's their stability. Our distinguished study is the investigation of functional ability of protease under different ranges of pH and thermo-stability along with their biochemical characterization of 70% ammonium sulfate precipitate protease enzyme preparation as well as its compatibility with various liquid and solid detergents and its application in blood de-staining performance from hospitalized bed cloth.

MATERIALS AND METHODS

Bacterial strain and protease production

Previously identified strong alkaline protease producer *Bacillus subtilis* IAS 01 (KF: 761633) from coastal region of rhizosphere soil was used for this study. This strain was cultured with respective substrates and fermentation conditions

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viz. Groundnut oil cake and palm oil cake in ratio of 5:5% (w/w), moisture 30%, 20% inoculum, pH 9.0 and temperature 37°C. After incubation period of 72 h, the cells were harvested at 10,000 rpm at 4 °C and the supernatant was used as further purification study.

Purification of protease and SDS-PAGE analysis

The clear supernatant was considered as the crude protease. This crude protease was precipitated with 70% ammonium sulfate to final concentration and the enzyme precipitate obtained by centrifuge at 10,000 ×g for 30 min. The solution was taken and dialyzed against minimum volume of buffer (25 ml of 0.05M Tris-HCl pH 8.0) dialyzed at 4°C against same buffer solution for 24 h in a large container and then the solution taken further filtration assay. The dialyzed enzyme was applied to DEAE-cellulose column (1.2 ×20 cm) equilibrated with 25 ml of 0.05M Tris-HCl pH 8.0. The unbound proteins were washed with same buffer and the bound proteins were eluted by a linear gradient with 0.5 M NaCl at the flow rate of 0.5 ml/min and each fraction (2.0 ml) was collected. Then the fractions were analyzed for protease activity and highly active fractions were pooled and concentrated and further applied on to gel filtration chromatography column contains Sephadex G-100.

The active fractions from DEAE-cellulose step, was desalted prior to loading into Sephadex G-100 column (2.6 cm × 90 cm) previously equilibrated with (25 ml of 0.05M Tris-HCl pH 8.0) and developed at a flow rate of 0.5 ml/min. Five ml of fractions were eluted with the same buffer and the eluted enzymes were concentrated and kept in an ice bath. Then the fractions were analyzed for protease activity and highly active fractions were pooled and concentrated and the molecular mass of the purified protease was determined through 15% SDS-PAGE, on a vertical gel apparatus.

Protease assay

Protease activity was quantitatively analyzed by the method of (Essakiraj *et al*., 2011) with using 1% casein as a substrate. Experimentally, 1 ml of culture supernatant, 0.5 ml Tris buffer (100 mM; pH 8.5) and 0.5 ml of 1% aqueous casein solution were added. The mixture was incubated for 30 min at 30°C. and add 3 ml 5% trichloroacetic acid into this mixture to terminate reaction,, whereby it formed a precipitate. The mixture was further incubated at 4°C for 10 min and then centrifuged at 10,000 rpm for 15 min. Thereafter, 1 ml supernatant was taken, to which 2 ml 0.5 M sodium carbonate was added, mixed well and incubated for 20 min. To this mixture, 0.5 ml Folin Ciocalteu Phenol reagent was added and the absorbance was read at 660 nm using a UV-Vis Spectrophotometer. A standard curve was constructed taking concentration of Tyrosine mg/ml on X-axis and corresponding optical density on Y-axis. Unit: One protease unit (PU) is defined as the amount of enzyme that released 1ug of tyrosine per ml per minute under the above assay conditions. The protein content in the samples was estimated by following the method of (Lowry *et al*., 1951). The amount of protein present in the sample was calculated from the standard curve.

Biochemical properties of the 70% ammonium sulfate precipitated protease

Effect of pH on protease activity and stability

To determine the optimal pH of the enzyme was measured over a range of pH (4.0-13.0) at 30°C with casein 1% (w/v) as substrate under standard assay condition except that pH was changed each step. The buffer system were as follows: 0.05M of citrate buffer (pH: 4.0-5.0), 0.05M potassium phosphate buffer for (pH: 5.0-7.0), 0.05M Tris-HCl buffer for (pH: 8.0–9.0), 0.05M Glycine-NaOH buffer for (pH: 9.0-11.0) and 0.05M sodium carbonate buffer for (pH 12-13). For the pH stability was subjected by pre-incubating the enzyme preparation at different buffer system ranging from 4.0-13.0 for 2 h 30°C and then the residual proteolytic activity was determined also under standard assay conditions.

Effect of temperature on protease activity and stability

The effect of temperature on protease activity was subjected from 30-75°C for 30 min at optimum protease pH by using 1% casein as a substrate and measured standard assay condition. The temperature stability of the enzyme was studied by incubating with same optimal pH at ranging from 50-70°C for 180 min and then residual activity was measured with every 30 min interval under standard assay condition. The non treated enzyme was taken as control (100%).

Effect of metal ions and inhibitors

In order to determine the effect of metal ions (5mM) concentration on enzyme activity were investigated by adding monovalent ions (Na^+ and K^+) and divalent ions (Mn^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+} and Cu^{2+}) to the reaction mixture, respectively. The effect of inhibitors on protease activity were investigated such as Phenylmethylsulfonyl fluoride (PMSF), Ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol (β-ME), dithio-bis-nitrobenzoic acid (DTNB) and soybean trypsin inhibitor (SBTI). The 70% ammonium sulfate precipitated enzyme was incubated with each inhibitor for 30 min at 30°C in the presence of 1% casein as a substrate. Subsequently, the enzyme activity was determined by the method previously described. The activity of the enzyme in the absence of inhibitors was taken as 100%.

Effect of surfactant and commercial detergent

In order to determine its stability in the presence of surfactants such as Sodium dodecylsulphate (SDS), Triton X-100, Tween 80 and oxidizing agent (H_2O_2) and sodium perborate ($NaBo_3$). The enzyme preparation was incubated with these additives for 30 min at 50°C and then the residual enzyme activities were determined under the standard assay conditions. Followed by further evaluated its compatibility of the enzyme with commercial solid detergents. The solid detergents such as Ariel, Tide, Henko, Rin and Surf excel were diluted in tap water to give a final concentration of 5 mg/ml. The endogenous enzymes in the detergents were inactivated by pre-incubating the diluted detergents for 1 h at 50°C prior to the addition of the protease enzyme and the residual enzyme activity was

determined under optimal assay conditions without any additive was taken as control.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 (SPSS, Inc., Chicago) statistical package. Data were expressed as men standard deviation (SD). One way analysis of variance (ANOVA) followed by Duncan multiple comparison method was used to correlate the difference between the variables. Data was considered statistically significant $p \leq 0.05$.

Wash performance analysis

Formulation of 70% ammonium sulfate precipitated protease was subjected blood de-staining assay. Experimentally dried blood stained cotton cloth (10 cm × 10 cm) were collected from Annamalai University, Rajah Muthiah Medical College Hospital. The following sets were prepared in tray. A) Tab water 100 ml + blood stained cloth as a control. B), Tap water 100 ml + blood stained cloth + 1 ml of commercial detergent (Surf excel-5 mg/ml). C) Tap water 100 ml + blood stained cloth + 0.05 M sodium carbonate buffer alone. D), Tap water 100 ml + blood stained cloth + 1 ml of precipitated protease (815.08±6.66 U/g) pH 12.0 maintained with 0.05M sodium carbonate buffer.

These experimental trays were incubated at 50°C for 60 min. The cloth pieces were taken out from each set at regular intervals of 15 min and then rinsed with water, dried and visually examined its quality.

RESULT

Protease purification

Alkaline protease was purified from the culture supernatant according to the procedure given in material methods section. To start with the purification steps, the cell-free supernatant obtained from the culture medium of *Bacillus subtilis* IAS 01 was precipitated with 70% ammonium sulfate in this step 2.5 fold purity was obtained. This protein concentrate was further purified by using DEAE sepharose Fast Flow column. The target protease was eluted between the concentration of 0.5M NaCl and in this step 6.17 fold purity was obtained. Then the highly active fractions were pooled and further purified with sephadex G-100, and in this step 11.63 fold purity was obtained when compared to crude culture filtrate. The summary of purification is also presented in (Table 1).

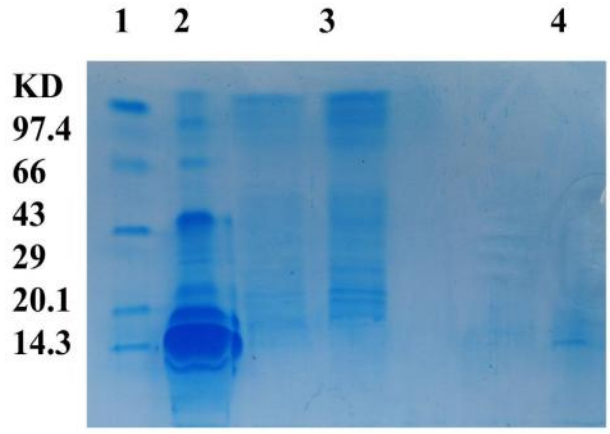

Fig1. SDS/PAGE result of purified protease from IAS 01. Lane 1: molecular mass markers; Lane 2: crude protease; Lane3: 70% ammonium sulfate precipitated protease Lane 4: purified protease.

Characterization of protease

Effect of pH and temperature on enzyme activity and stability

The protease from IAS01 was highly active in the pH range of 6.0–13.0 around 4.0-13, with an optimum level revealed that the pH 12.0 (Fig, 2).

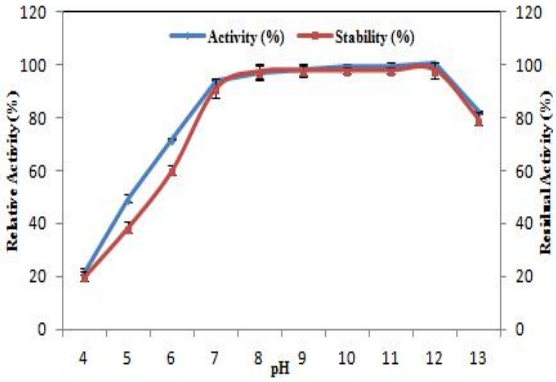

Fig 2 Enzyme activity was assessed through the range of 4-13 using specific pH buffer solution. The maximum enzyme was highly activity at pH 8.0-12.0 and it was considered as 100% activity. Stability was determined by incubating the enzyme in different buffer for 1h at 40°C and the residual activity was measured at pH 12.0 and 60°C.

Interestingly is an essential characteristic for the use of alkaline protease as detergent and de-haring additives. The pH stability of the (precipitated) protease was also stable at wide range of pH 6.0-13. Temperature activity was analyzed at different temperature ranging from 30°C-75°C by constant pH 12.0.

Table 1 Summary of the protease purification by *Bacillus subtilis* IAS01

Purification step	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein concentration (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	100	835.13	83513	6.86	686	121.74	100	1
Ammonium sulfate (70%)	37	1132.53	41903.61	5.71	211.27	198.34	50.18	2.5
DEAEcellulose	21	942.75	19797.75	2.95	61.95	319.58	23.71	6.17
Sephadex G-100	10	872.86	8728.6	1.95	19.5	447.62	10.45	11.63

The purified protease by *Bacillus subtilis* IAS 01 was also confirmed to be single purified protein molecule by using SDS-PAGE and its molecular weight was determined as 14.3 kDa by using proteins of known molecular weight (Fig,1).

An experimental results the *Bacillus subtilis* IAS01 protease activity was found to be an optimum temperature at 55°C and also showed activities over wide range of temperature 45°C-70°C (Fig,3). In order to determine thermo-stability of the prepared enzyme was incubated for 180 min at pH 12.0 at

different temperatures ranging from 50°C-70°C. In order to determine the protease stability is concerned, the enzyme retained 100% activity in the temperature range of 50–55°C resulted in (Fig 4).

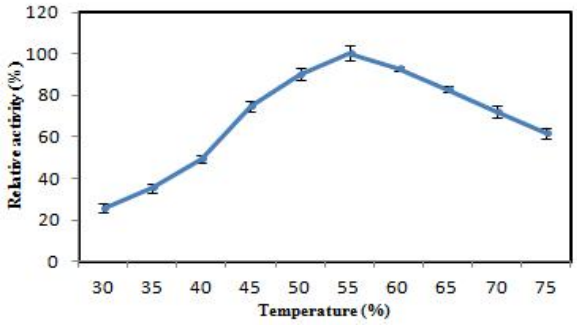

Fig 3 Temperature profile was determined at different temperature between 30-75°C. The activity of enzyme was optimum at 55°C consider as 100%. Bars are indicated standard deviation and each value concluded out six replicates

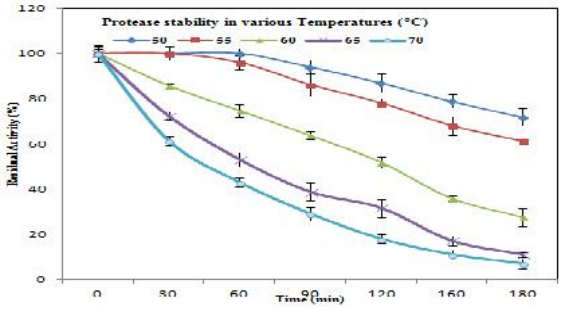

Fig 4 Thermo-stability of the ammonium sulfate precipitated enzyme was determined by incubating enzyme at different temperatures from 50-70°C for 180min. The residual activity was measured at each 30min under the standard assay condition. The original activity was before pre- incubate was taken as 100%. Bars are indicated standard deviation and each value concluded out six replicates.

Effect of various metal ions and inhibitors on the protease activity

In order to determine the effect of metal ions in protease activity was studied at concentration of 1 mM. Result showed in (Table 2), the precipitate protease activity was found to be highly active in the presence of Ca^{2+} (130.88±1.48) followed by Mg^{2+} (114.57±1.30) and Cu^{2+} (103.48±1.31), respectively. The effect of a various enzyme inhibitors on enzyme activity were examined and resulted in (Table 3).

Table 2 Effect of metal on protease activity

Metal ion	Concentration (mM)	Relative activity (%)
control	0	100
Mg^{2+}	5	$114.57±1.30^{b}$
Ca^{2+}	5	$130.88±1.48^{a}$
Mn^{2+}	5	$89.65±1.95^{d}$
Cu^{2+}	5	$103.48±1.31^{c}$
Zn^{2+}	5	$65.82±1.85^{f}$
Na^{+}	5	$76.42±1.41^{e}$
K^{+}	5	$65.82±1.85^{f}$

IAS01 (70% ammonium sulfate precipitated) protease was pre-incubated with different metal ions for 1 h at 50°C, and the activity was calculated under standard assay condition and without addition of metal was taken as 100%. Value are represent mean of six replicates and significantly analyzed with respect p≤0.05.

Table 3 Effect of inhibitors on protease activity

Inhibitors	Concentration (mM)	Relative activity (%)
Control	**0**	100
EDTA	1	$5.83±0.64^{e}$
PMSF	1	$95.10±0.51^{b}$
β-Mercaptoethanol	1	$97.79±4.95^{a}$
DTNB	1	$84.70±1.34^{c}$
SBTI	1	$72.95±1.25^{d}$

IAS01 (70% ammonium sulfate precipitated) protease was pre-incubated with various inhibitors for 1 h at 50°C, and the residual activity was measured under standard assay condition and without addition of additive was taken as 100%. value represent as mean ± standard deviation for six replicates.

The proteolytic activity was strongly inhibited by EDTA (1mM) with reduced the original activity (94.2%), followed by serine protease inhibitors (PMFS) and exhibited 95% of its original activity was recorded. Therefore it was this suggested that the alkaline protease from IAS01 was belonging to the group of alkaline Endopeptidases like metalloprotease.

Effect of surfactant and commercial detergent

The enzyme preparation was incubated for 30 min at 50°C in the presence of surfactant/oxidizing agents such as SDS, Tween 80, Triton X-100, sodium perborate and H_2O_2. After an experimental result, the enzyme was relatively stable with 0.5 (w/v) anionic surfactant like SDS result shown in (Table 4), on other hand non ionic surfactant such Triton x-100 and Tween 80 1% (v/v) retaining more than 95% of its original activity, respectively. Furthermore, the less stable against an oxidizing agent as retaining 88% and 84% of its original activity after 30 at incubation of 50°C untreated enzyme was consider as 100%. To investigate stability of the alkaline protease with washing commercial laundry detergents, the enzymatic preparation was pre-incubated in the presence of various detergent for incubate at 50°C keep 1 hour. After experimental assay the stability of the protease was highly stable with all tested detergent result shown in Table-3. An interestingly the alkaline metalloprotease was highly stable in Surf-excel at 50°C and retaining 97% of its original activity followed by Ariel, respectively.

Table 4 Effect of Surfactants and oxidizing agents on protease activity

Surfactants/oxidizing agents	Concentration (%)	Residual activity
None	0	100
SDS	0.5 (w/v)	60.73±1.0
Tween 80	1 (v/v)	95.36±5.0
Triton X-100	1 (v/v)	94.21±1.3
Sodium perborate	1 (w/v)	88.22±0.6
H_2O_2	0.2 (v/v)	84.86±1.0

IAS01 (70% ammonium sulfate precipitated) protease was pre-incubated with various surfactants and oxidizing agents for 30 min at 50°C, and the residual activity was measured under standard assay condition and without addition of additive was taken as 100%. The activity is expressed as a percentage of activity level in the absence of additives. Data represent as mean ± standard deviation for six replicates.

De-staining assay

The 70% ammonium sulfate precipitated protease (pH 12.0 maintained with 0.05M Na_2Co_3) was investigated for its application in blood stain removal from hospitalizes bet cloth. The blood stained cloth pieces were soaked in enzymatic preparation and commercial detergent with various stages

mentioned in material methods section. An experimentally revealed that the (1132.53±6.66 U/ml) precipitated protease was completely removed the blood stain within 30 min compared to solid detergent (surf excel) us well as control (Fig 5).

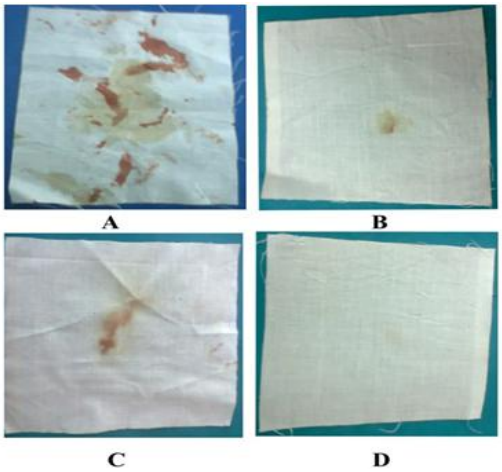

Fig 5 Application of 70% Ammonium sulfate precipitated protease on blood de-staining performance. A, Cloth washed with tap water. B, Cloth washed with surf-excel (5mg/ml). C, Cloth washed with 1 ml 0.05 M sodium carbonate buffer alone. D, Cloth washed with 1 ml enzyme preparation (pH maintained with 0.05 M sodium carbonate buffer) alone.

Table 5 Effect of commercial detergents on protease activity

Detergent	Weight (mg)	Residual activity (%)
None	0	100
Arial	5	75.45±1.19
Rin	5	88.90±1.13
Henko	5	64.6±1.39
Surf-Excel	5	97.76±0.70
Tide	5	79.32±1.0

IAS01 (70% ammonium sulfate precipitated) protease was pre-incubated with various commercial detergents for 1 h at 50°C, and the residual activity was measured under standard assay condition and without addition of additive was taken as 100% . The activity is expressed as a percentage of activity level in the absence of additives. Data represent as mean ± standard deviation for six replicates.

DISCUSSION

Industrially important extracellular alkaline protease was production from *Bacillus subtilis* IAS01by using SSF with suitable culture condition. The clear supernatant was consider as alkaline protease and could be purified followed by the methods such as 70% ammonium sulfate precipitation, DEAE cellulose and sephadex G 100 column with respective purification fold (11.63) and its yield 10.11%, respectively. Similarly observation found to be purification with sephadex G-200 obtain the yield 5.6% from *Bacillus* sp. B001 described by (Wen *et al*., 2010). The purified protease by IAS 01 was also confirmed to be single purified protein analysis by using SDS-PAGE and its molecular weight was determined as 14.3 kDa by using standard known molecular weight. Similarly reported by (Essakiraj *et al* 2013) low molecular weight protease with 18.3 kDa in *Bacillus subtilis* AP-MSU6 and they also obtained 23.99 fold of purity in Sephadex G-75 gel filtration chromatography. Another observation made by (Nasri *et al*., 2009) documented the related molecular weight of surfactant stable alkaline serine protease from Bacillus mojavensis A21 and its molecular weight of the purified enzyme was estimated to be 20 kDa by SDS-PAGE and gel filtration. Biochemical nature of 70% ammonium sulfate protease was determined such as optimum pH, temperature activity and stability, metal ion, inhibitor and stability of surfactant and commercial detergent. To find out the optimum activity pH: 8.0-12 and stability 12.0 was recorded among pH 3.0-13.Interestingly is an essential characteristic for the use of detergent formulation and dehairing process. These findings are in accordance with earlier reports showing the optimum pH 10.0-10.5 reported by (Banik *et al*., 2004). Moreover in this result 'IAS01' protease preparation was clearly indicated is more efficient than other alkaline protease from *Bacillus licheniformis* P003 reported by (Sarkar *et al*., 2013). The enzyme was exhibited for optimum temperature activity at 55°C and stability range of 50–55°C. Similarly described by (Vijayaraghavan *et al*., 2012) the optimum temperature of alkaline protease was most efficient than detergent-stable dehairing protease from *Bacillus subtilis* vv. During the study indicated that decrease in relative activity of precipitated protease was subjected to above 55°C for might be due to the autolysis or denaturation of the enzyme at higher thermophilic temperature and its stability might be an advantage for using this protease in detergents formulation at tropical places. Optimum activity of metal ion was exhibited Ca^{2+} (130.88±1.48) at concentration of 1mM, EDTA (1mM) inhibits the 94.2% of its original activity and it was concluded alkaline type of metalloprotease. In accordance with similar study described by (Nelson *et al*., 2004) EDTA was significantly inhibit the protease activity and it recorded as metalloprotease and another report described as based on the similar study (Olajuyigbe *et al*., 2014). Recoded the stability of surfactant was highly stable with 0.5 (w/v) anionic surfactant like SDS, non anionic surfactant such as Triton x-100 and Tween 80 1% (v/v) retaining more than 95% of its original activity and commercial detergent was highly stable in Surf-excel at 50°C and retaining 97% of its original activity. Alkaline metalloprotease from IAS 01 was successfully removed the blood stain from cloth within 30 min. These results are highly significant other observation of *B. licheniformis* MP1 (Jellouli *et al*., 2011) MP1 protease was effectively remove the blood stain from white cloth with 1 h 7mg/ml of Axion laundry detergent supplemented with MP1 crude protease. Another researcher *(Jayashree et al., 2012)* reported by the thermostable alkaline protease from alkaliphilic Strain of *Bacillus pumilus* MCAS 8 stain removed from fabric after 10 min of incubation at room temperature without the aid of detergents. Therefore, the results clearly indicated that preparation of enzyme with 0.05M sodium carbonate was significantly enhanced blood stains removal from cotton cloth and these properties clearly indicated its possible way of use in detergent formulation. Furthermore in this method new way to reduce the soil pollution compared to commercial detergent.

CONCLUSION

In conclusion, the characteristics of 70% ammonium sulfate precipitated enzyme was highly active and stable at high pH and temperature, it showed stability towards both ionic, non ionic surfactant and various commercial detergent. Furthermore, 1% EDTA inhibited the protease activity indicated as alkaline type of metalloprotease and could

effectively remove the blood stain from hospitalized cloth pieces. Considering its promising properties IAS01 enzymatic preparation we suggest that better choice for bio detergent.

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