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Full Length Research Paper

Purification and Characterization of 56 KDa cold active Protease from *Serratia marcescens*

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The extracellular cold active protease produced from *Serratia marcescens* TS1. The protease was purified to homogeneity from the production medium by ammonium sulphateation then followed by acetone precipitation with 80% saturation. The cold active protease was fractionized by diethylaminoethyl (DEAE) cellulose column chromotography. The molecular weight of protease was approximately 56 KDa. The isoelectric point was close to 6.4. The maximal activity towards casein was found at 40°C and its pH activity was at 8.0. The protease was strongly inactivated by $HgCl_2$ metal ion and reactivated by $FeSO_4$ thus indicated as metalloprotease. The protease was inhibited by Na_2 ethylenediaminetetraacetic acid (EDTA). The protease of *S. marcescens* TS1 showed a potential application in the laundry industry by removeing the blood, chocolate and egg youlk stains from the white cotton cloths in a short period without changing texture of cloths.

Key words: Cold active Protease, *S. marcescens* TS1, extracellular protease, metalloprotease, laundry application.

INTRODUCTION

Serratia marcescens is a gram-negative bacteria belonging to the genus *Serratia* and family enterobacteriaceae (Grimont and Grimont, 2006). The protease secreted by *Serratia marcescen* was purified by thin layer chromatography (Matsuyama et al*.,* 1986). The advance in biotechnological techniques and enzyme engineering paves way for industrial application of protease (White et al*.,* 1973)**.** Proteolytic enzymes from microorganisms may be located within the cell or excreted into the media (Kohlman et al*.,* 1991). Proteases added to laundry detergent enable to release the proteinaceious materials from stained cloths (Masse and Tilburg, 1983). In addition it improves washing efficiency allows shorter period of agitation, often after a preliminary period of soaking (Nielsen et al*.,* 1981; Demidyuk et al., 2008). Many other keratinolytic alkaline proteases were used in feed technology for the production of peptides for degrading waste keratinous materials in bathtub and drains in public places (Takami et al., 1992). It is now firmly established that enzymes in organic solvents can expand the applications of biocatalysts in synthetic chemistry (Zaks and Kilbanov, 1984; Zaks, 1991). Some studies have demonstrated the possibility of using alkaline protease to catalyse peptide synthesis in organic solvents (Golobov et al., 1994). The mechanism in each case is the ability of an enzyme to cleave or cut proten target in to two or more pieces usually at a very specific cleavage sites (Mazzone et al., 1990).

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MATERIALS AND METHODS

Bacterial strains

The psychrotrophic bacterial strain *S. marcescens* TS1 screened from the soil of dense apple garden around Badran Magam in Ksahmir at altitude of 1630 meters above the sea level. The bacterial strain was grown at 15°C for 24 h in casein enzyme hydrolysate medium (Rifaat et al., 2007). The strain TS1 has the high proteolytic activity was identified by morphological and biochemical test. Then confirmed based on 16s ribosomal deoxyribonucleic acid (rDNA) gene sequence which were submitted to the Gene Bank and compared with other bacteria by

phylogenetic analysis.

Enzyme production

The cold active protease was produced by following a method of Salamone and Wodzinski (1997) using the enzyme production medium Tryptone-yeast extract glucose broth containing: Tryptone 5 g/L, Yeast extract 2.5 g/L, Glucose 1 g/L and pH 7.2. For the study of protease production 250 ml of medium was poured into 1000 ml of Erlenmeyer flask capacity, were sterilized at 121°C for 15 min.

After cooling 0.5 ml of stationary phase culture of strain TS1 was inoculated and incubated on shaker at 28°C for 48 h.

Enzyme purification

The cold active protease was purified by following method of Matsumato et al. (1984) from culture broth by centrifuged at 8,944 ×g for 20 min at 4°C. The supernatant was collected and filtered through membrane filter having porosity of 0.022 µm at 4°C. To the supernatants ammonium sulphate was added slowly with continuous string to the final concentration of 80% saturation. The enzyme solution was allowed to stand for 24 h at 4°C and centrifuged at 8,944 ×g for 20 min. The precipitate was resuspended in 50 mM Tris HCl having pH 8.0 and further precipitated with acetone by adding slowly to the final concentration 80% saturation and left for 1 h at 4°C. The pellet was obtained by centrifugation at 8,944 ×g for 20 min at 4°C and resuspended in 20 mM Tris HCl pH 8.0 then dialyzed against 500 ml of 5 mM Tris-HCl pH 8.0 containing 1 mM $MgCl_2$ over night at 4°C with stirring conditions. The dialyzate was centrifuged at 5,724 ×g for 20 min at 4°C and supernatant were subjected to diethylaminoethyl (DEAE) - cellulose anion exchange column chromatography equilibrated with 10 mM Tris-HCl buffer pH 8.3. The 15 ml of dialysate eluted with 10 mM Tris-HCl buffer pH 8.3 at the flow rate of 20 ml/h. A linear gradient consisting of 50 ml of 10 mM Tris-HCl buffer pH 8.3 and 50 ml of the same buffer with 0.3 M NaCl. The 5ml of fractions elute was collected and absorbance measure at 280 nm and enzyme activity was determined.

Determination of protein content and assay of proteolytic activity

The protein concentration of strain TS1 was determined by the method of Lowry et al. (1951) by taking bovine serum albumin as standard. The proteolytic activity was determined by following a method of Kunitz (1947) using casein as substrate. The substrate contained 3.75 ml of 1.0% casein in 100 mM Tris-HCl and 1 mM $MgCl_2$ at pH 8.0. The 0.5 ml of protease sample was added to the substrate and incubated for half an hour at 30°C. After incubation, the reaction was quenched with 0.5 ml of 10% trichloro acetic acid. The quenched reaction mixture was centrifuged at 10000 rpm for 10 min to pellet precipitated protein and absorbance for the supernatant was determined at 280nm. One unit of proteolytic was defined as the amount of enzyme that produced an increase of absorbance at 280 nm of 0.1 under the conditions of the assay.

Molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of the protein in strain TS1 was determined by the method of Laemmli et al. (1970) staining the protein with 10% methanol, 7% acetic acid and 0.2% coomassie brilliant blue for 4 h and destained with 10% methanol, 25% acetic acid solution for 12 h. The molecular weight analysed by calculated the distance travelled by the protein marker and distance travelled by the sample.

Isoelectric focusing of protein

The isoelecric focusing of purified protease of strain TS1 was determined using mini-gel system (Robertson et al., 1987)**.** The gel was placed in staining solution for 30 min and destained for one hour. The bands were observed in white light transilluminator.

Effect of temperature on protease activity

A 0.2 ml of cold acive protease of strain TS1 was added to the substrate mixture containing 1.5 ml of 1.0% (w/v) casein in 100 mM Tris-HCl in 1 mM $MgCl_2$ at pH 8.0 and incubated at 25, 30, 35, 40, 45, 50, 55, 60°C for 1 h. After the incubation, the proteolytic activity was determined by the protease assay, an optical density was measured at 280 nm.

Effect of pH on protease activity

A 0.2ml of cold acive protease of strain TS1 was added to the substrate mixture containing 1.5ml of 1% (w/v) casein, and 0.1 mM $MgCl_2$ in various buffers. Such as Glycine-HCl buffer having pH 2.0, 2.5, 3.0, 3.5, Acetate buffer having pH 4.0, 4.5, 5.0, 5.5, phosphate buffer having pH 6.0, 6.5, 7.0, Tris- HCl buffer having pH 7.5, 8.0, 8.5, 9.0 and carbonate bicarbonate buffer having pH 9.5, 10.0, 10.5, 11.0 and incubated at 37°C for 60 min. After incubation the proteolytic activity was determined by the protease assay.

Effect of metal ions on protease activity

A 0.2 ml of cold acive protease of strain TS1 was added to the 1.5 ml of 0.1 M Tris-HCl pH 7.5 and to the same buffer supplemented with 100 µl of 8.3 mM of metal ions viz $MgSO_4$, $MnCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$ and $ZnCl_2$ and mixtutes were incubated at room temperature 25°C for 30 min and proteolytic activity was determind by protease assay. In addition the purified protease sample preparation (200 µl/ml) was incubated for 30 min at 25°C in 0.1 M acetate buffer having pH5 5.0 supplemented with (100 µl/ml) Na_2EDTA and protease activity was determined by protease assay.

Effect of inhibitors on protease activity

A 0.2 ml of cold acive protease of strain TS1 preparation was added in to 1.5 ml of 0.1 M tris- hydrochloride buffer having pH 7.5 and to the same buffer supplemented with 100 µl of various inhibitors 20 mM Na_2EDTA, 8.3 mM iodioacetic acid, 8.3mM dithiothreitol, 8.3 mM leupeptin, 1% of 2 β-mercaptoethanol, 1% of tween-20 and 3% of ethanol and mixtutes were incubated at 25°C for 30 min and proteolytic activity was determind by protease assay.

Assessment of detegent additive role of protease in laundry industry

The application of cold active protease strain TS1 as detergent addivites in the laundry industry (Masse and Tillburg, 1983) was carried out by taking 100µl/ml of protease strain TS1 and 200mg/ml of wheel detergent on white cotton cloth pieces (10 ×10 cm) stained with human blood, chocolate and egg yolk. The stained clothes incubated at room temperature for 4 h and washed with water

Table1. Purification of Protease from *S. marcescens* TS1 in the supernatants of tryptone yeast extract glucose medium.

S/N	Purification Stage	Volume (ml)	Protein conc. protein (mg/ml)	Total (mg)	Activity (U/ml)	Specific activity (U/mg)	Total Recover activity (U)	Purification fold	(%)
1	Cell free culture supernatant	2500	0.9	2250	864	960	2160000	1	100
2	Ammonium Sulphate fraction	200	4.5	900	7890	1753.3	1578000	1.9	92
3	Acetone fraction	50	5.9	295	23800	4033.8	1190000	4.2	70
4.	Dialysis	100	0.6	60	3950	6583.3	395000	6.9	60
5.	DEAE cellulose Fraction	50	0.3	15	2840	9466.6	142000	9.9	51

then result was noted.

RESULTS

Bacterial soil isolate

The total 211 strains were isolated from the soil and the potential cold actine proteolytic strain TS1 was found gram negative rod shaped bacterium, non-flagellated, non motile, non endospore former. The colony morphological appreance found red pigmented convex, transparent in nature. The biochemical tests showed indole negative, methyl red negative, vogues proskauer positive and citrate positive, bacteria does not produce hydrogen sulphide gas. 16s rDNA gene sequences confirmed that it belongs to *S. marcescens* therefore this bacterium named as *S. marcescens* TS1 under Gene Bank ACC. No. GU046543.

Enzyme purification

The purification process showed that 80% ammonium sulphate saturation had precipitated the protease in the solution by salt out mechanism and further recovered with 80% acetone saturation. The dialysed cold active precipitated protease of *S. marcescens* strain TS1 fractionalised by DEAE Cellulose anion exchanged chromatography with 10 mM Tris HCl buffer pH 8.3 were shown in Table 1.

Determination of protein content and proteolytic activity of protease enzyme

The proteolytic activity was 83.84 IU/ml in casein as substrate. The molecular weight of protease *S. marcescens* strain TS1 was found approximately 56 KDa protein band when observed under white transilluminator (Figure 1) and isoelectric point was 6.4 in an ampholyte buffer having pH ranges from 2.0 to 11.0 (Figure 2).

Effect of temperature on protease activity

The maximium temperature for the cold active protease of *S. marcescens* TS1 was 20°C in 100 mM Tris HCl buffer as shown in Figure 3. The activity declined rapidly above 25°C and was negligible above 50°C. The enzyme retained its 82% activity at 25°C when temperature increased the enzyme activity decreases rapidly and lost at 50°C.

Effect of pH on protease activity

The hydrogen ion concentration of cold active protease *S. marcescens* strain TS1 was 8.5 with a sharp decrease in activity above pH 9.0. The protease had half maximal activity near pH 7.5 and exhibited a little activity below pH 3.5. The protease retained its maximum activity from pH 6.5 to 9.0 (Figure 4).

Effect of metal ions on protease activity

The metal ions have altered the protease activity of *S. marcescens* strain TS1. The $HgCl_2$ and Na_2EDTA have inactivated the protease at both pH 8.5 and pH 6.5. The protease have retained maximum activity in $FeSO_4$, $MgSO_4$, $ZnCl_2$ and minimum activity in $MnCl_2$, $CaCl_2$, $CuSO_4$ and lost its activity in $HgCl_2$ and Na_2EDTA (Table 2). The data indicates that cold active proteases of the *S.*

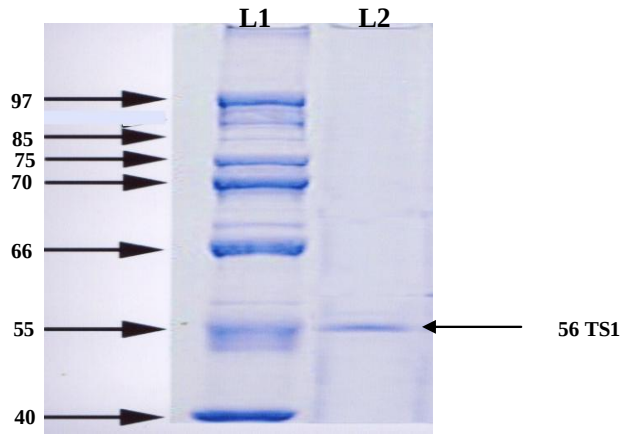

Figure 1. Determination of molecular weight of proteases by sodium dodecyl sulphate agarose gel electrophoresis**. L1—** Molecular marker mass standards: phosphorylase b (97 kDa), tyrosine (85 kDa), acid phosphate (75 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa) and aldolase (40 kDa), **L2**--Protease sample of *S. marcescens* TS1 (56 kDa).

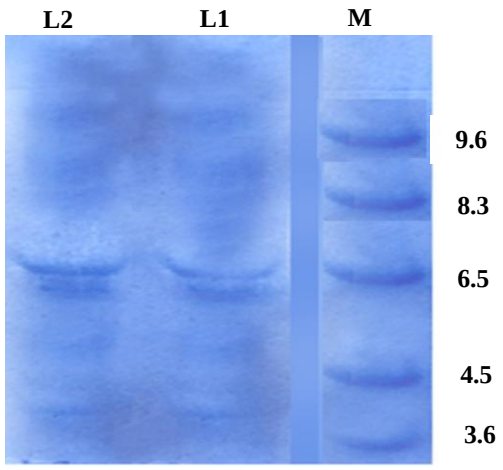

Figure 2. Isoelectric focusing electrophoretogram, pH 2.0 to 11.0 stained with coomassie blue. **M**—Isoelectric focusing standards: amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.5), carbonic anhydrase II (pI 6.5), lentil lectin (pI 8.3) and ribonuclease A (pI 9.6), **L1 and L2--***Serratia marcescens* TS1 showing pI 6.4.

marcescens strain TS1was to be a metalloprotease because inactivated by the Na_2EDTA and reactivated by the Mg^{2+}, Fe^{2+}, Zn^{2+} is due to increase in the absorbance value at 280 nm and their residual enzyme activity.

Effect of inhibitors on protease activity

The cold active protease of *S. marcescens* strain TS1

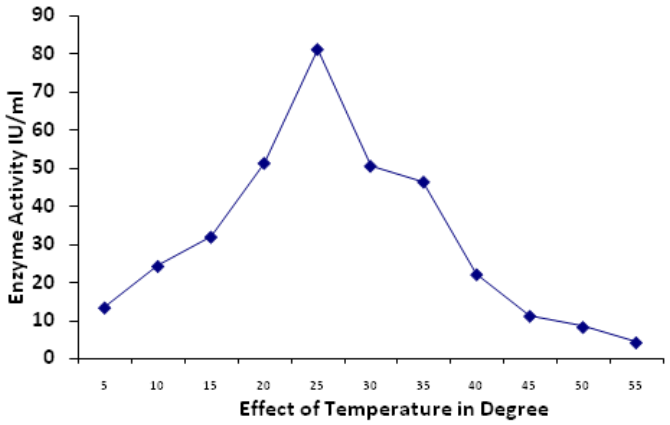

Figure 3. Effect of Temperature on protease activity was examined in 100 mM Tris-HCl buffer having pH 8.0 at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55°C for 30 min. The *Serratia marcescens* strain TS1 showed maximum activity at optimum 25°C.

showed the resistant against the all inhibitors except 20 mM EDTA. The protease retained 80% activity in iodioatic acid, 83% activity in 2-mercaptoethanol, 90% activity in tween 20, 87% activity in 3% ethanol, 73% activity in leupeptin but lost its activity in 20 mM EDTA (Table 3).

Detergent application

There was the litte blood stain in the white cotton cloth which was treated with detergent only but the blood stain was completely removed from the white cotton cloths which were treated with both detergent and protease of *S.marcescens* strain TS1. In case of chocklate and egg yolk there was a stains even when treated with detergent but completely removed when treated with both detergent and protease of *S. marcescens* strain TS1. Thus indicated that protease in presence of detergent removed the stains completely from white cotton cloth pieces (Figure 5).

DISCUSSION

The *S. marcescens* strain TSI secreates large extracellular enzyme protease in the surrounding medium (Yanagida et al., 1988). The production was stoped at early stationnary phase at that time maximium protease was produced (Henriette et al., 1993). The 80% ammonium sulphate saturation leads the precipitation of the protease at 4°C and fractional precipitation with acetone (Salamone and Wodzinski, 1997). The excess salt removed from protease by means of a dialysis (Morita et al., 1997). The dialyzate of *S. marcescens* strain TS1 purified by ion exchange chromatography

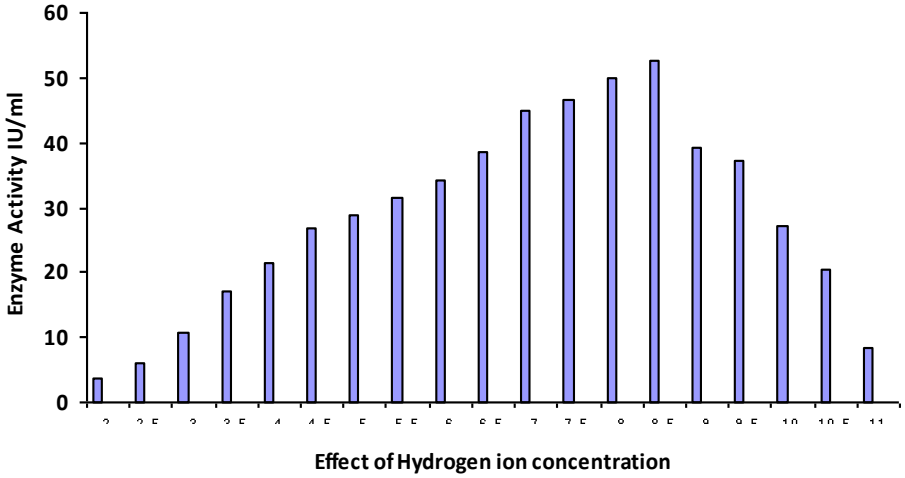

Figure 4. Effect of pH on protease activity was examined in various buffers such as Glycine-HCl buffer having pH 2.0 to 3.5, Acetate buffer having pH 4.0 to 5.5, Phosphate buffer having pH 6.0 to7.0, Tris-HCl buffer having pH 7.5 to 9.0 and Carbonate-Bicarbonate buffer having pH 9.5 to 11.0 at 30°C for 30 min. The pH optimum of *Serratia marcescens* strain TS1 was at pH 8.5 Tris-HCl buffer.

Table 2. Effect of metal ions on protease activity was examined in 8.3 mM of $MgSO_4$, $MnCl_2$, $CaCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$ and $ZnCl_2$ in 0.1 M Tris-HCl buffer having pH 7.5 at 25°C for 30 min. The metal ions $HgCl_2$ and 20 mM Na_2EDTA have inactivated the protease of *Serratia marcescens* TS1.

S/n	Metal ion	Residual protease activity (%)
1	Native protease	100
2	$FeSO_4$	81
3	$MnCl_2$	22
4	$CaCl_2$	15
5	$CoSO_4$	11
6	$ZnCl_2$	70
7	$MgSO_4$	78
8	$HgCl_2$	00
9	Na_2EDTA	00

Table 3. Effect of inhibitors on protease activity was examined in 20 mM EDTA, 8.3 mM Iodioacetic acid, 8.3 mM Dithiothreitol, 8.3 mM Leupeptin, 1% of 2- β Mercaptoethanol, 1% of Tween 20 and 3% of ethanol in 0.1 M Tris-HCl having pH 7.5 at 25°C for 30 min. The protease of *Seratia marcescens* strain TS1 was inactivated completely by 20 mM EDTA.

S/N	Inhibitor	Residual protease activity (%)
1	Native protease	100
2	Iodioacetic acid	80
3	2- Mercaptoethanol	83
4	Tween 20	90
5	3% Ethanol	8
6	Dithiothreitol	73
7	Leupeptin	82
8	20 mM EDTA	00

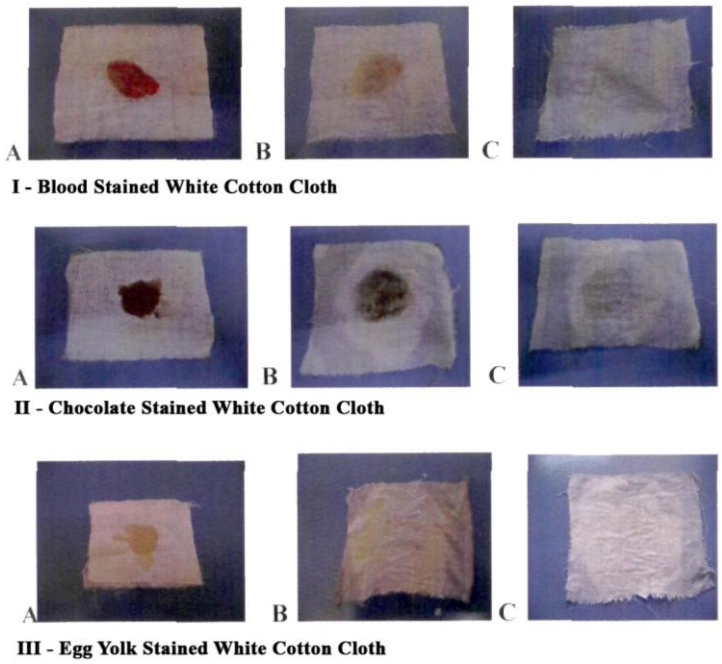

Figure 5. Washing test of protease *Serratia marcescens* strain TS1.
1. A. Blood stained cotton cloth, **B.** Washed with detergent wheel only, **C.** Washed with both wheel detergent and protease of *Serratia marcescens* TS1
II. A. Chocolate stained cotton cloth, **B**. Washed with wheel detergent only, **C.** Washed with both protease of *Serratia marcescens* TS1 and wheel detergent.
III. A. Egg yolk stained cotton cloth, **B**. Washed with wheel detergent only, **C.** Washed with both protease of *Serratia marcescens* TS1 and detergent.

relied on the attraction between oppositely charged particles. The net charge exhibited by these compounds was depends on their pka and pH of the solution. The proteolytic activity was determined by using casein as substrate (Kunitz, 1947) in Tris HCl buffer pH 8.0 showed the protease activity of 83.84 IU/ml. The purified exocellular protease turned out to be one polypeptide chain with a molecular weight of 56 KDa averages of the values obtained by SDS-PAGE (Laemmli, 1970). The isoelectric point of protease *S. marcescens* strain TS1 was 6.4 (Robertson et al., 1987). As proteins are differing in the composition each and every protein has its own characteristic p^{I} value. The optimal timperature of protease *S. marcescens* strain TS1was 40°C in Tris HCl buffer containing $MgCl_2$ having pH 8.0. The protease activity lost when temperature increased at 60°C there was neglible activity. The protease activity was neglible when temperature increased from mesophilic bacteria is around 60°C (Boguslawski et al., 1983). A psychrotrophic *Pseudomonas fluorescens* 114 produced a protease with an optimal temperature of 35°C (Hamamoto, 1994), 37°C an antarctic yeast (Ray et al., 1992) and 40°C by psychrophilic *vibrio* sp. strain 5709 (Hamamoto et al., 1995). The pH characteristics of cold proteases *S. marcescens* strain TS1 showed high enzyme activity between 6.5 to 9.0 and maximum at 8.5 in Tris HCl buffer (Lyerly and Kreger, 1979). The metals ions $HgCl_2$ and Na_2EDTA completely inactivated the protease activity (Matsumoto et al., 1984) and reactivated by Mg^{2+}, Fe^{2+}, Zn^{2+} and Mn^{2+} are essential for the enzyme activity so named as metallprotease (Aiyappa and Haris, 1976). The 20 mM EDTA inhibited the enzyme activity completely while as other inhibiters did not showed much impact on enzyme activity. The potease of *S. marcescens* TS1 acted on the stains and degraded the protein bonds among the proteins present in the blood, chocolate, and egg yolk (Masse and Tilburg, 1983). The increased usage of these proteases as detergent additives is mainly due to the cleaning capabilities of these enzymes, environmentally acceptable and non phosphate detergents.

Conclusion

The cold active protease of *Serrratia marcescens* strain TS1found to more active to remove the dirts and stains from the clothes at low temperature in a short time

without damaging the nature of the cloth. So the economic values and enhencer actions will be boom to the detergent industry as detergent additives.

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