ISSN 2319 – 7757 EISSN 2319 – 7765

Indian Journal of Engineering

Optimization, purification and characterization of alkaline protease enzyme from *Streptomyces indicus* var.GAS-4

Guravaiah M[1]*, Hatti I, Prabhakar T, Daniel K, Sirisha P, Nautha Mary T, Anusha A, Jahnavi N

1. Department of P.G Biotechnology. Jagarlamudi Kuppuswamy Choudary College, Guntur-530 003, A.P, INDIA
2. Department of Zoology. Jagarlamudi Kuppuswamy Choudary College, Guntur-530 003, A.P, INDIA
3. Department of Chemistry. Jagarlamudi Kuppuswamy Choudary College, Guntur-530 003, A.P, INDIA
4. Andhra University College of Pharmaceutical Sciences,Vizag-530017, A.P, INDIA

Received 28 October; accepted 21 November; published online 01 December; printed 16 December 2012

ABSTRACT

An alkaline protease producing actinomycete strain *Streptomyces indicus* (GAS-4) was isolated from the soil samples collected from various places at Guntur, Prakasam and Khammam districts of Andhra Pradesh, India and was optimized under submerged conditions. Maximum enzyme production of the culture occurred at 37^0C and pH9.0. The molecular weight of the enzyme determined by SDS-PAGE was found to be 60kDa.Higher alkaline protease activity was observed at an optimum temperature of 37^0C and pH-9 using glycine-NaOH buffer. PMSF was able to inhibit the enzyme completely.Ca^{+2} and Na^+ metal ions addition resulted in increased protease activity of enzymes.The protease showed a high level of hydrolytic activity against casein and moderate hydrolysis of BSA and egg albumin. The enzyme retained more than 50% activity with most of the detergents (Ariel, Surf Exel, Mr.White, Tide, Rin, Wheel) tested even after 3 hr incubation at 37°C after the supplementation of $CaCl_2$ and glycine. The supplementation of the enzyme preparation in detergent i.e. Ariel could significantly improve the cleansing performance towards the blood stains.

Keywords: Submerged fermentation, SDS-PAGE, hydrolytic activity, detergent.

1. INTRODUCTION

Submerged fermentation is one of the method in which the isolated actinomycete strain was grown in the selected production medium and its activity is estimated. A total of nine variables were selected and were used for Optimization studies by Design of Experiment (DOE). These include: pH, Inoculum, Temperature, RPM, Age of the inoculums, Incubation period, Glucose, Yeast extract and Tryptone. Purification techniques employed were: Ammonium sulphate precipitation, Sephadex G-200, IEC and Gel filtration. The alkaline protease activity was determined by each purification technique and the desirable process was selected. Novel methods like Native PAGE and Zymography were also carried out. The details of the methods were given below and their results were graphically determined.

2. MATERIALS AND METHODS

Isolation of Actinomycete strain

An alkaline protease producing actinomycete strain was isolated from soil samples collected from various places at Guntur, Prakasam and Khammam in Andhra Pradesh. Culture was maintained on starch casein agar slants at 4°C.

Preparation of cell suspension

The inoculum was prepared and used for the fermentation.

Optimization of cultural parameters

Factors such as temperature, pH, incubation time, age and level of inoculum, vitamins amino acids trace elements, metal inhibitors and surface active agents which influence the secretion of protease enzyme by actinomycetes were optimized for maximum production. Experiments were conducted in Erlenmeyer flasks (250ml) containing production medium [(g/L): glucose, 10; soya bean meal, 20; $CaCl_2$, 0.4 and $MgCl_2$, 2] and incubated at 28°C on incubator shaker for 96 h (180 rpm). After fermentation, 5 ml broth was centrifuged at 3000 rpm for 10 minutes and assayed for enzyme activity. The experiments were carried out in triplicate and the average values are-presented. Protease activity was estimated by the method of Lowry et al., (1951).

Effect of various temperatures on alkaline protease production

The production flasks were incubated at 25°C, 26°C, 28°C, 30°C, 32°C, 35°C and 37°C for 96 h.The results are presented in Fig.9.

Effect of various initial pH values on alkaline protease production

The production medium was adjusted at pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0). The results are presented in Fig.10.

Effect of various incubation periods on alkaline protease production

The flasks with the production medium (pH 9.0) were inoculated and incubated at 37°C. Samples were withdrawn periodically at every 12 h up to 96 h and assayed for protease activity. The results are presented in Fig.11.

Effect of level of inoculum on alkaline protease production

Experiments were carried out using 2.0%, 3.0%, 5.0% and 7.0% inoculum volume each containing $6.24xl0^6$ spores/ml. The production medium (pH 9.0) flasks were inoculated and incubated at 28°C for 96 h. (Fig.12).

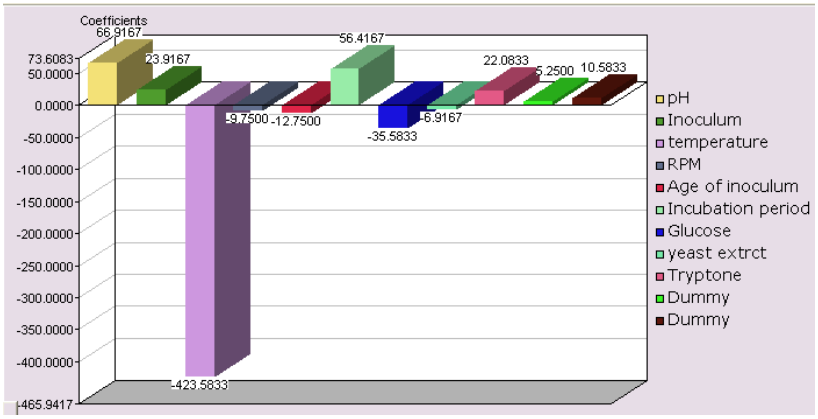


Figure 1

Histogram showing various contributing factors

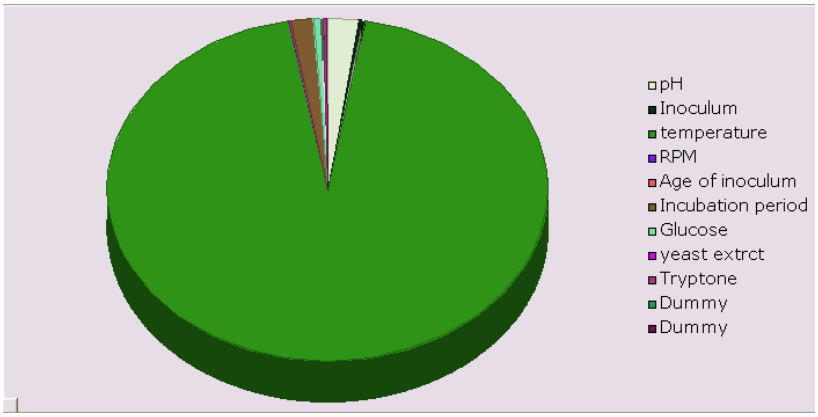


Figure 2

Pie diagram showing various contributing factors

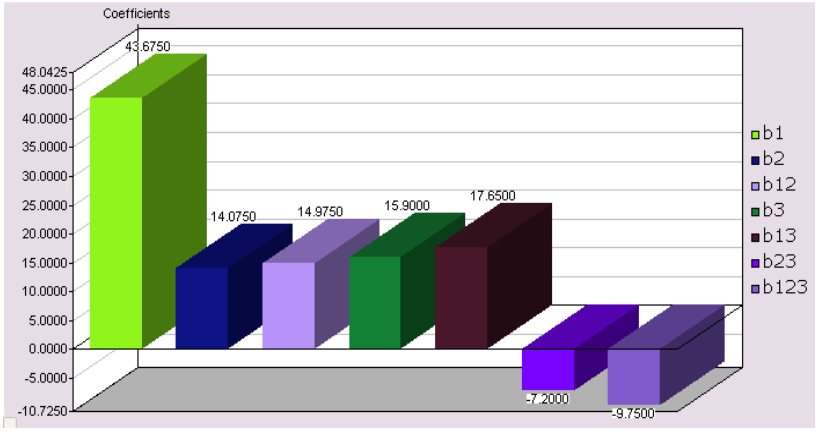


Figure 3

Graphical display-Histogram

Effect of age of inoculum

The flasks with the production medium were inoculated using cultures of different age (24, 36, 48, 60, and 72 h old culture as inoculum) at 10% level and incubated at 28°C for 96 h. The results are presented in Fig.11.

Effect of addition of various amino acids on protease production

Factors like amino acids are known to influence the production of microbial metabolites (Malabika, 2001). The fermentations were conducted at pH 10 in the basal production medium [(g/L) glucose, 10; soya bean meal, 20; CaCl2, 0.4; $MgCl_2$, 2] at 28^{0}C for 96h. The fermentation broths were centrifuged and were assayed for alkaline protease activity.

Effect of amino acids on protease production

In the present work, the effect of different amino acids viz., D-alanine, DL-alanine, L-alanine, L-arginine, L-asparagine, L-cysteine, L-cysteine HC1, glycine, L-glutamic acid, L-glutamic acid sodium salt, L-leucine, L-lysine, L-histidine, L-tryptophan and L-tyrosine on protease production was studied.The solutions of individual amino acids were at a concentration of 0.5%. A control was run using water. Protease activities were determined. The results were given in Fig.13.

Experimental design and optimization by rsm for the strain *Streptomyces indicus* var. GAS-4

Estimation of alkaline protease activity:

Alkaline protease activity was assayed by method of Lowry *et al*., (1951). Optimization of the process by applying DOE (Design of Experiments) statistical techniques was done. The process involved 9 variables. They are: pH, Inoculum, Temperature, RPM, Age of inoculum, Incubation period, Glucose, Yeast extract and Tryptone (Table 1). As per Paretos Law, when there are many variables, it is the vital few significant factors that affect the process rather than many trivial factors and this process is screening. Therefore a Placket Burman, a statistical technique has been used which offers only 12 experiments for 9 variables. For this purpose software SigmaTech has been used for statistical planning and analysis to screen the effective process variables (Table 2).

Placket Burmann Design plan and observations of experiments (Table 2)

Inference it can be seen from the observations that whenever temperature is at 28^{0}C, the enzyme concentration is the highest. The above data was used for statistical analysis by ANOVA Table 3. The analysis as given above is explained in Table 4 with comments and analysis of the above result is:

1) Temperature is contributing to the output increase very significantly since its contribution is 94.51% in terms of SS%.
2) If One percent of contributing variables are selected for optimization of the process, only pH, temperature & Incubation period can be only three parameters for optimization.
3) All other variables having negative coefficient can be kept as constant at lower levels where as variables of positive coefficient can be kept as constant at higher level so that some effect of these variable though small can be available for the process.

Histogram

Histogram **(**Fig.1**)** indicates that only temperature, inoculum, and pH are only contributing factors. Thus out of 9 process variables, only three are the variables that have significant effect on the process output and hence they only planned for optimization. In both the pictures (Fig.1) & (Fig.2) only temperature is one single contributing factor. But in order to see the further scope finally 3 variables have been selected for optimization with Factorial Design of Experiments a part of RSM. In fact next optimization can be done only changing the temperature to a lower level. How ever since the experimentation is to be done to verify to increase the output, we can as well try with three variables as shown Table 5. A software SigmaTech has been used for statistical planning and analysis. The factorial design of the Experimental Plan is given in Table 6. As per this the experiments were conducted and the outputs are recorded in the Table 7. Statistical analysis of this data is given in the Table 8.

Graphical display

The graphical display in the form of Histogram (Fig.3) and Pie diagram (Fig.4) were shown.

Inference

Temperature X1 has the highest positive coefficient and also highest SS% contribution. However the optimum temperature determined was 28^{0}c with further increasing temperature protease yield decreased. All variables have positive coefficient and therefore it calls for increase in variables to boost up

Guravaiah et al.
Optimization, purification and characterization of alkaline protease enzyme from *Streptomyces indicus* var.GAS-4,
Indian Journal of Engineering, 2012, 1(2), 108-117,

output. The observations indicate that at higher temperature, increase in pH or Incubation period will offer higher output. Therefore the process model has been simulated.

The process model:
Y^= b0 +b1X1 +b2X2 +b3 X3+ b12 X1X2 +b13X1X3 + b23 X2X3 + b123 X1X2X3
By substituting the coefficients from the above table in place of bs the process model can be described as follows.
Y^= 63.875 +43.675 X1 + 14.075 X2 + 15.9 X3 +14.975 X1X2 + 17.65 X1X3 - 7.2 X2X3 – 9.75 X1X2X3
Where,
Y^ = estimated value of output
X1= Temperature.
X2= pH
X3= Incubation period in hrs

Verification of model accuracy

By substituting the above planned variables in this model, we get the Y^ i.e. estimated output (Table 9). It can be seen that the estimated values match with actual observations by which we can infer that it is perfect model. The highest output is 153.2 U/ml which is most satisfactory. Thus just 12 experiments with Plackett Burman method and 8 experiments as per Factorial with Design of Experiments we could obtain give a maximum output of 153.2U/ml.

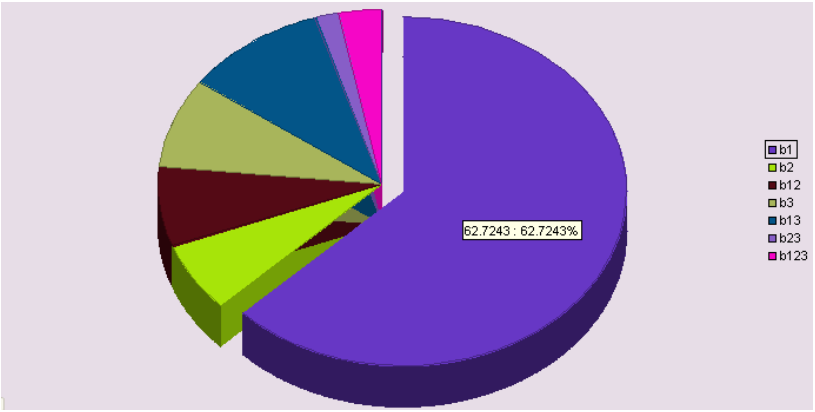


Figure 4
Graphical display-Pie diagram

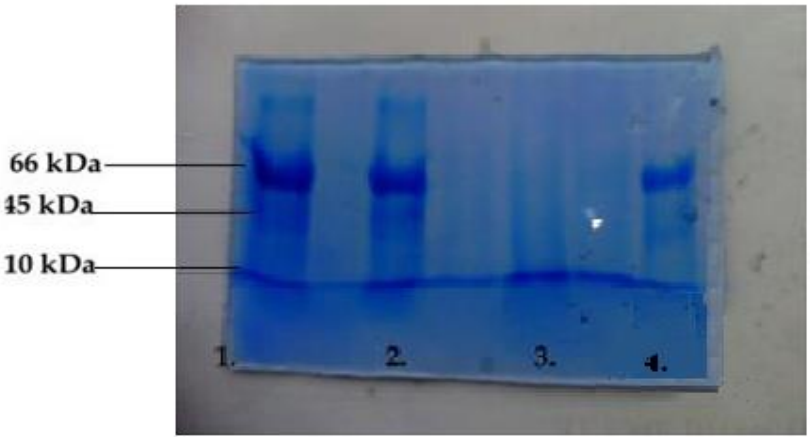


Figure 5
SDS-PAGE of alkaline protease from *Streptomyces indicus* GAS-4

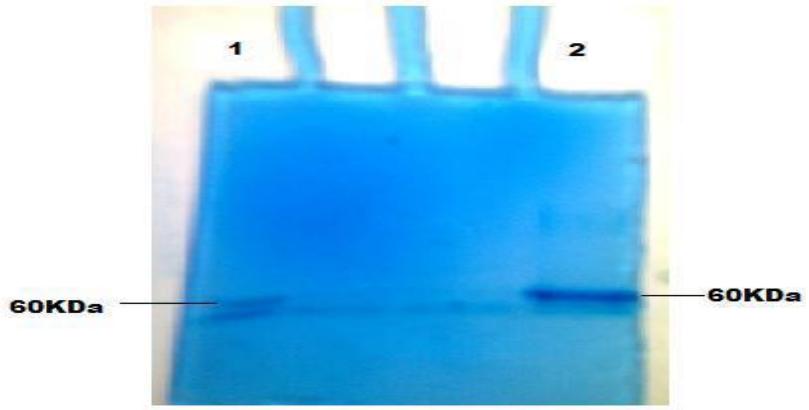


Figure 6
Native PAGE of alkaline protease from *Streptomyces indicus* GAS-4

Production of alkaline protease in a 2litre fermenter

The alkaline protease production was carried out in a 2 litre fermenter (B.Braun Biotech International, Micro DCU-200) containing 1.5 L modified production medium. The composition of modified production medium was (g/L): Glucose, 10; Soyabean meal, 20; $CaCl_2$, 0.4; $MgCl_2$, 2.0 with pH 10. A 10% (v/v) level of inoculum was added and the fermenter was run at 37^{0}C for 48 hours. After the completion of fermentation, the whole fermentation broth was centrifuged using Sor vall RC 5C centrifuge at 10,000rpm at 4^{0}C and the clear supernatant was separated. The supernatant (crude enzyme) was subjected to recovery and purification process.

Enzyme recovery and purification procedure

Ammonium sulphate precipitation

To obtain complete precipitation of the crude enzyme, the supernatant was subjected to ammonium sulphate precipitation at 60% saturation. For this purpose, solid ammonium sulphate (195g) was added gradually with mechanical stirring to harvest fluid (2x500ml) at4^{0}C to a saturation of 60%. The precipitate so formed was separated by centrifugation (8000g) for 15min., resuspended in cold saline solution (100ml) and dialyzed in cold against 1L of 0.05M Tris-HCl-0.1M NaCl (pH 10) for 20 hrs. After dialysis, the solution was centrifuged and supernatant obtained was designated as fraction –I. It was used for Zinc Sulphate precipitation.

Zinc sulphate precipitation (Step II)

Fraction- I obtained after Ammonium sulphate precipitation was subjected to Zinc Sulphate precipitation. About 100ml of the pooled supernatant (fraction -I) with activity of 20U/mg protein was diluted with 1.4 L of 0.05M Tris-HCl-0.1M NaCl (pH 10) to obtain 2 units of absorbance at 280nm. To this solution 75ml of 0.1M Zinc sulphate was added drop wise with stirring at 0^{0}C in an ice bath. The precipitate thus formed was separated by centrifugation (8000g) for 15min., and suspended in 50ml of 0.4M Sodium citrate. This solution was dialyzed in cold (4^{0}C) against 240ml of 0.05M Tris-HCl-0.1M NaCl, pH 10. The resulting solution was designated as fraction-II and was subjected to gel filtration chromatography.

Sephadex G-200 gel filtration chromatography (step III)

The dialyzed enzyme (fraction-II) was centrifuged at 8000g for 15min. and supernatant was chromatographed on a column of Sephadex G-200. The sample (fraction-II) was loaded on to a column of Sephadex G-200 (1.5cmx24cm) equilibrated with 0.05M Tris-HCl-0.1M NaCl (pH10). The column was eluted at a slow rate of 1.0ml/hr with a discontinuous gradient from 0.1M to 0.8M NaCl in the same buffer. A total of 40 fractions were collected. A typical chromatogram is shown. From the elution profile it was observed that the protein was eluted as a well resolved peak of Caseinase activity coinciding with single protein peak at a NaCl concentration of 0.4M. Fractions (15-18) with high protease activity were pooled together, dialyzed and concentrated by lyophilization and

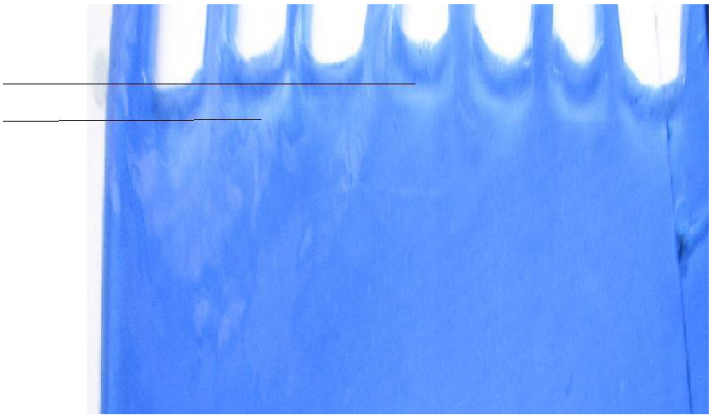


Figure 7
Zymograph of alkaline protease from *Streptomyces indicus* GAS-4,
The arrow mark showing colourless patches is the indicative of protease

used for further studies. It was labeled as fraction-III.

ION-EXCHANGE CHRAMOTOGRAPHY

The dialyzed enzyme subjected to ion exchange chromatography containing DEAE-Cellulose .the resin was poured to the column and equilibrated with 10mM Tris –Cl buffer (Ph-7.0).the dialyzed sample was loaded to the column and its was eluted from column by using gradient elution.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

After Sephadex G-200 column chromatography, the fractions (15-18) showing the highest specific activity was dialyzed, lyophilized and then subjected to SDS-PAGE. The SDS-PAGE was performed according to Laemmli (1970) using 10% acrylamide (Fig.5).

Native PAGE

Here the gel electrophoresis is run in the absence of SDS and DTT. The electrophoretic mobility in SDS-PAGE depends on the molecular mass, while in native PAGE the mobility depends on both protein's charge and its hydrodynamic size (Deyl, Z., 1979). Native PAGE serves as an excellent tool to study conformation, self-association or aggregation, and the binding of other proteins or compounds in neutral pH conditions (Hames, B.D., 1990). Thus it is a powerful technique to study structure and composition of proteins since both conformation and biological activity remain intact during the process. (Laemmli., 1970). The results were shown in Fig.6.

ZYMOGRAPHY METHOD

Zymography is the latest method being used to analyze Matrix Metalloproteinases (MMP) and Tissue Inhibitors of Metalloporteinases (TIMP) in biological samples (Fig.7). This technique is rather simple, sensitive, accurate and quantifiable. In zymography, the proteins are separated by gel electrophoresis where separation occurs in polyacrylamide gel (Patricia A.M. et al., 2005). Alkaline protease activity of the purified enzyme was determined.

Protein assay

Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was calculated from the absorbance at 280 nm.

Determination of kinetic parameters of the purified alkaline protease from the strain gas-4

Effect of substrate concentration on Alkaline protease activity

In order to characterize the alkaline protease produced by the strain of GAS-4, the enzyme (1mg/ml) was incubated at a time interval of 30 min with different concentrations of protease. The protease concentration was shown in Table 11 and Fig.15.

Characterization of purified enzyme

Effect of pH on purified enzyme activity and stability:

Activity of the purified protease was measured at different pH values to study the effect of different pH values on activity. The pH was adjusted using the following buffers (0.05 M): phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0) and glycine-NaOH (pH 9.0-12.0). Reaction mixtures were incubated at 55°C for 30 min. and the activity of the enzyme was measured. The results are shown in Fig.14. To determine the stability of enzyme at different pH values, the purified enzyme was diluted in different relevant buffers (pH 6.0-12.0) and incubated at 37°C for 2 and 20 h. The relative activity at each exposure was measured as per assay procedure and the results are shown in Fig.14.

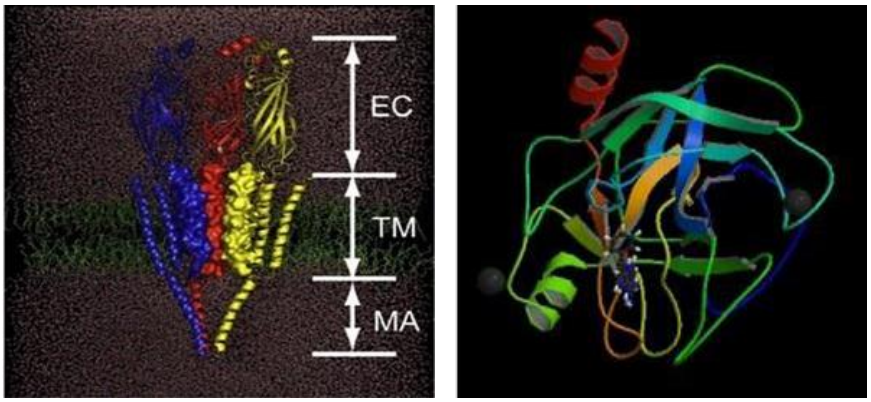


Figure 8
(A) Production template; (B) 3D Protease respect to template; (C) Observed structure of protease

Effect of temperature on enzyme activity and stability:

The activity of the purified enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30 to 100°C for 30 min. in the absence and presence of 10 mM $CaCl_2$. The results are shown in Fig.16. To determine the enzyme stability with changes in temperature, purified enzyme was incubated for 30 min. at different temperatures (60, 70, 80 and 90°C) in the presence of 10 mM $CaCl_2$ and relative protease activities were assayed at standard assay conditions. The results are shown in Fig.17.

Effect of protease inhibitors and chelators on enzyme activity:

The effect of various protease inhibitors (at 5mM), such as serine inhibitors [Phenylmethylsulphonyl fluoride (PMSF) and Diisopropyl fluorophosphate (DFP)], cysteine-inhibitors [p-chloromercuric benzoate (pCMB) and β-mercaptoethanol (β-ME), and a chelator of divalent cations [Ethylene diamine tetra acetic acid (EDTA)] were determined by preincubation with the enzyme solution for 30 min at 37°C before the addition of substrate. The relative protease activity was measured. The results are shown in the Fig.18.

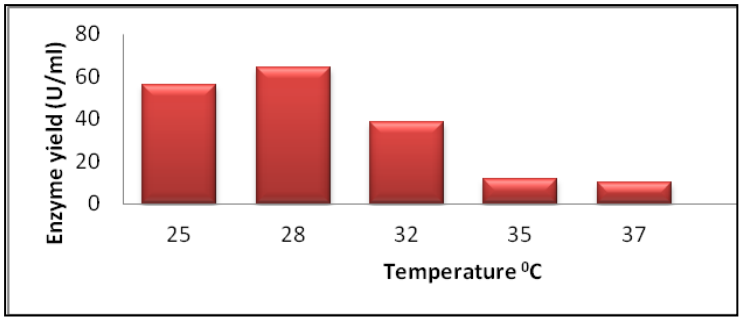


Figure 9
Effect of initial temperature on enzyme production

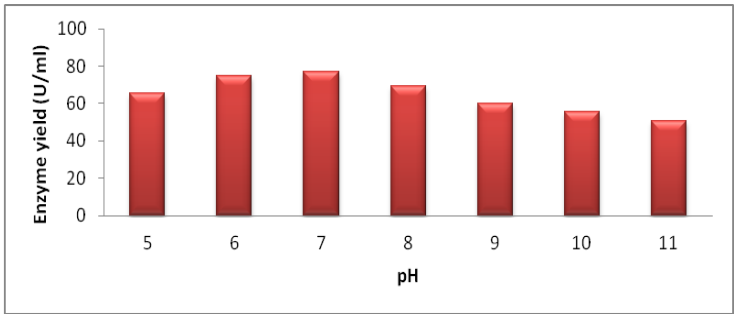


Figure 10
Effect of initial pH on enzyme production

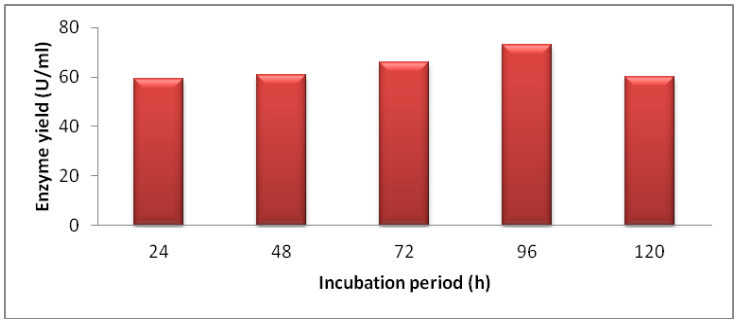


Figure 11
Effect of incubation time

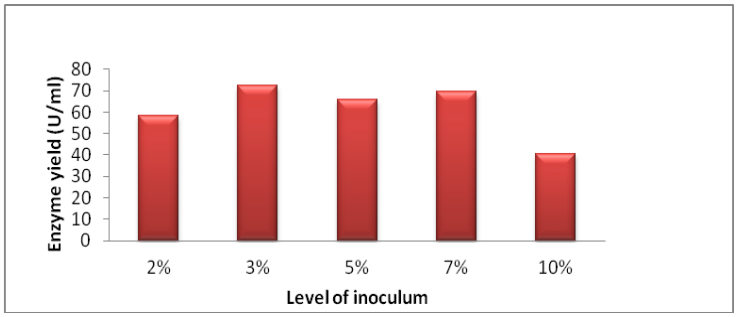


Figure 12
Effect of inoculum level on enzyme production

Effect of various metal ions on protease activity:
The effects of different metal ions viz., Ca^{2+}, Mg^{2+}, Co^{+2}, Cd^{+2}, Fe^{+3}, Na^{+}, Zn^{2+} and Cu^{2+} (10 mM) were investigated by adding them into the reaction mixture. The mixture was incubated for 30 min. at 37°C and the relative protease activities were measured. The results are shown in the Fig.19.

Hydrolysis of protein substrates
Protease activity with various protein substrates such as bovine serum albumin (BSA), casein, egg albumin and gelatin was assayed by mixing 100ng of the purified enzyme and 200µl of assay buffer containing the protein substrates (2 mg/ml). After incubation at 37°C for 30 min, the reaction was stopped by adding 200µl of 10% TCA (w/v) and allowed to stand at room temperature for 10 min. The undigested protein was removed by centrifugation and the released peptides were assayed. The specific protease activity towards casein was taken as a control. The results are shown in the Fig.20. The compatibility of our enzyme was studied with Ariel in presence of 10 mM $CaCl_2$ and 1M glycine for different time periods (0.5–3h) at 60°C. The results are shown in Fig.23.

Washing test with protease preparation
Application of protease as a detergent additive was studied on white cotton cloth pieces (4 × 4 cm) stained with blood (0.1ml) and kept aside for 1h. The stained cloth pieces were taken in separate flasks. The following sets were prepared and studied:

1. Flask with distilled water (100 ml) + stained cloth (stained with blood).
2. Flask with distilled water (100 ml) + stained cloth (stained with blood)
   + 1 ml Ariel detergent (7mg/ml).
3. Flask with distilled water (100 ml) + stained cloth (stained with blood)
   + 1 ml Ariel detergent (7mg/ml) + 2 ml enzyme solution.

The above flasks were incubated at 37°C for 15 min. After incubation, cloth pieces were taken out, rinsed with water and dried. Visual examination of various pieces exhibited the effect of enzyme in removal of stains (Fig.22). Untreated cloth pieces stained with blood were taken as control.

Dehairing of animal skin with protease
The dehairing capacities of the crude broth and purified enzyme were studied on fresh goat skins. For this purpose, enzyme solutions (20 ml) were applied as a paste with kaolin (10g) and streptomycin sulphate (100mg) on the flesh side of freshly slaughtered paired goatskin pieces. The skins were kept aside for 6h. A control was also kept using water instead of enzyme solution. After 6h contact time, the ease of dehairing was noted by removing the hairs with a blunt scalpel. The results are shown in Fig.21.

3. RESULTS & DISCUSSIONS

Effect of incubation temperature
The results are shown in Fig.9. The results indicated that the organism grew over a wide range of temperatures (25^0 to 37^0C). At 37, 45 and 45^0C, the protease production was 109, 148 and 204U/ml respectively. The maximum alkaline protease production (222U/ml) was observed at 37°C at 96 h. Increase in incubation temperature to 60°C decreased the yield to 186 U/ml. Hence the optimum incubation temperature is 37°C for pure enzyme.

Effect of initial pH
The highest yield (226.2 U/ml) was shown at pH 10.0. So the optimum pH for protease production was found to be 9.0.

Effect o f incubation time
The highest yield (224 U/ml) was achieved at 96 h. After that the protease production decreased gradually with increased incubation periods.

Effect of age of inoculum
The results from Fig.11 indicate that culture of 96 h age had maximum protease producing ability (72.8 U/ml)

Effect of level of inoculum
The results in Fig.12 indicate that protease production was increased with increase in level of inoculums upto 10% level (230 U/ml) and further increase in inoculums level decreased the protease production.

Effect of Amino acids on protease production
The Fig.13 indicates that many of the amino acids (at 5µg/ml concentration) tested were shown stimulating effect on protease production. Among them, L-cysteine had shown maximum (198%) increase in protease production. Stimulating effect of amino acids are in the order of L-cysteine > L-tyrosine, L-tryptophan (176% increase) > L-lysine HCl, L-cysteine HCl (48% increase) > L-alanine (25% increase) > DL-alanine (18% increase) > L-arginine, L-aspargine (16% increase).

Guravaiah et al.
Optimization, purification and characterization of alkaline protease enzyme from *Streptomyces indicus* var.GAS-4,
Indian Journal of Engineering, 2012, 1(2), 108-117,

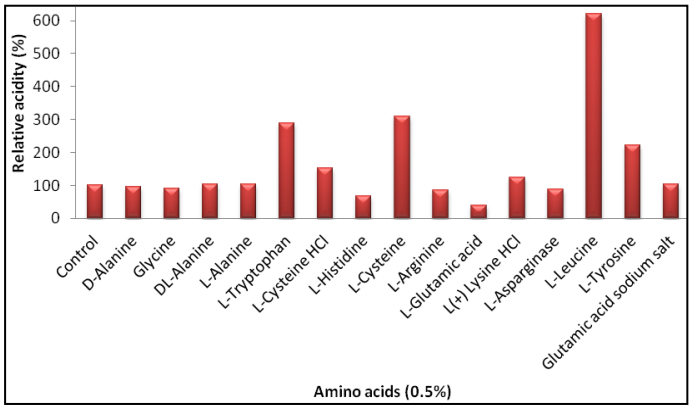


Figure 13
Effect of amino acids on protease production

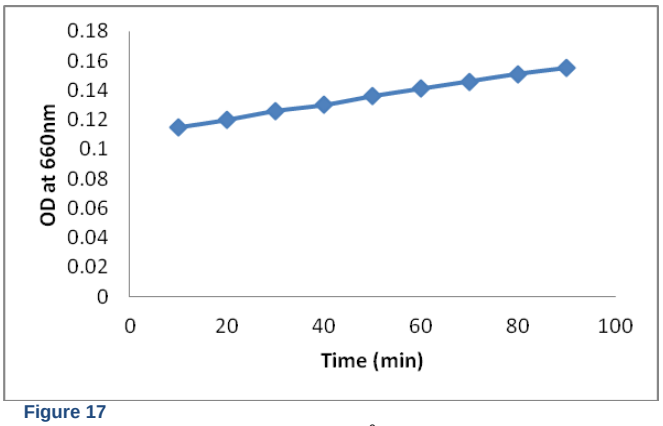
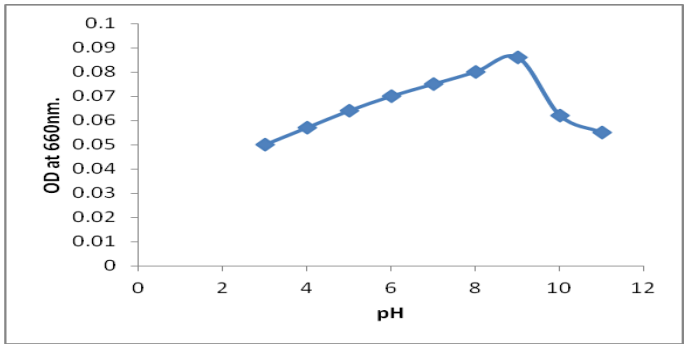


Figure 17
Effect of temperature on the stability at 37^{0}C



0Figure 14
1Effect of pH on the activity of alkaline protease

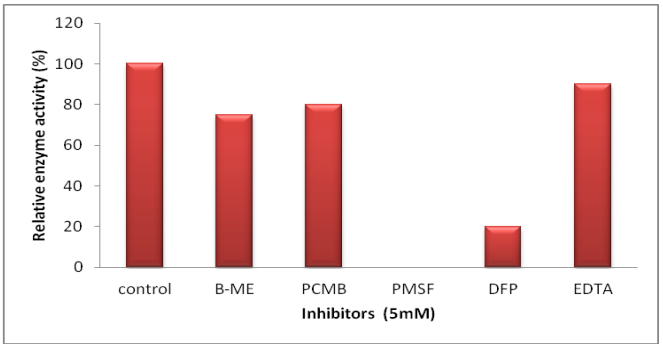


Figure 18
Effect of protease inhibitors/chelators on enzyme activity

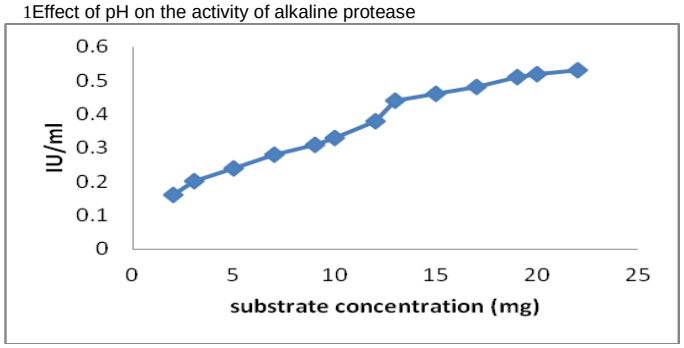


Figure 15
Effect of substrate concentration on protease activity

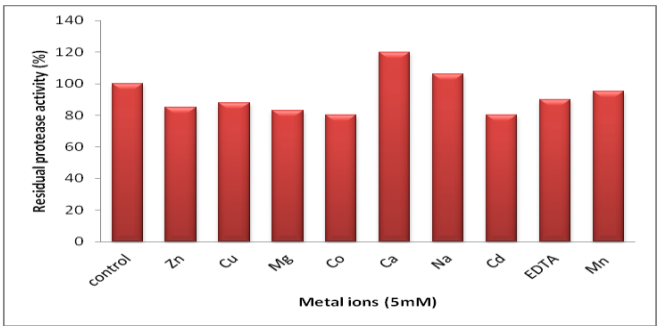


Figure 19
Effect of various metal ions on alkaline protease activity

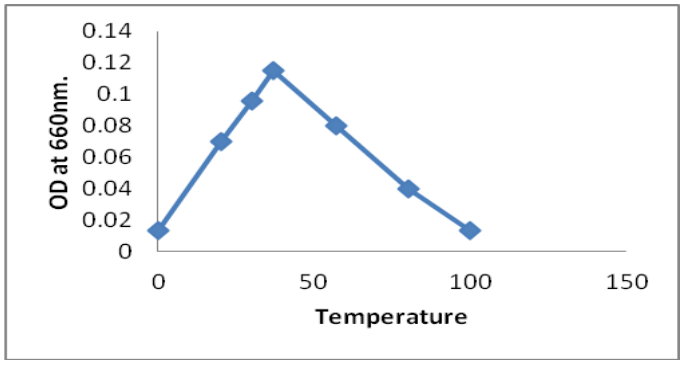


Figure 16
Effect of temperature profile on the activity of alkaline protease

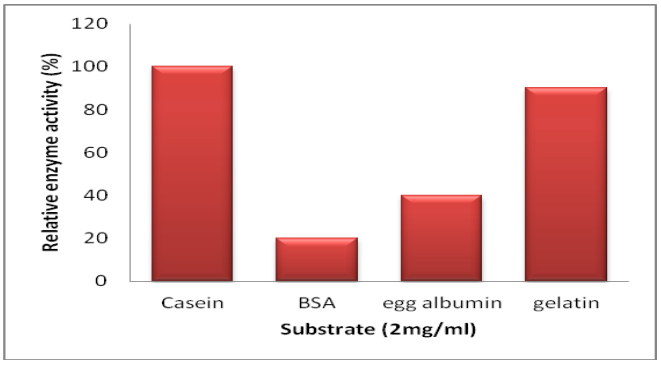


Figure 20
Alkaline protease activity against different natural substrates

3.1. Results and discussion of RSM

The promising isolate GAS-4 was used for protease production which was carried out in four modified production media. Maximum protease was obtained in a medium containing Glucose 1%, Yeast extract 0.5% and Tryptone 0.5. Nine variables were selected and protease production at two levels (Lower level, higher level) was carried out according to the Placket-Burman design in 12 experimental runs. Maximum protease 1448.0 U/ml was present in run no. 5. Statistical analysis by ANOVA revealed temperature is contributing 94.5% in terms of SS%. pH, Inoculum and temperature are the other variables whose contribution is greater than 1% and hence were selected for further optimization. Later a factorial design of the experimental plan was made for these three variables (temperature, pH, incubation period) at three levels (Lower level, midpoint and higher level). Highest protease 153.2U/ml was

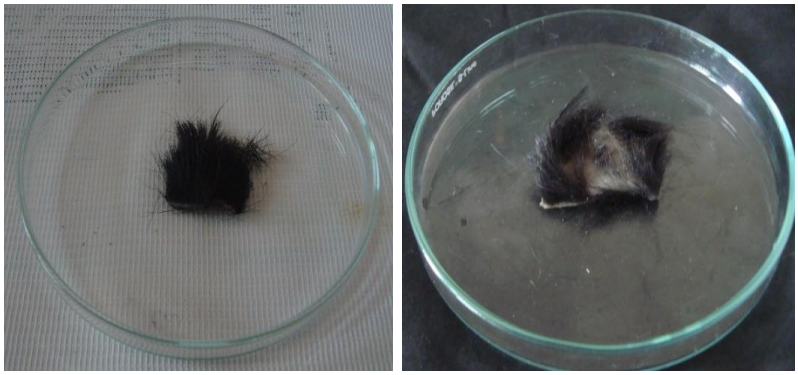


Figure 21
Comparision of proteolytic activities on dehairing of goat skin before (A) and after (B) addition of the enzyme

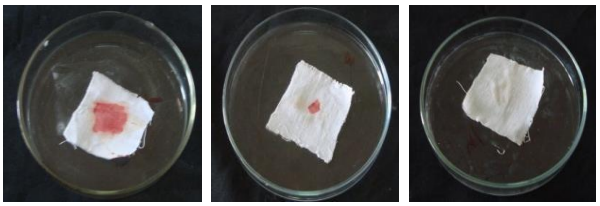


Figure 22
Washing performance of alkaline protease from *Streptomyces indicus* GAS-4 in the presence of detergent (Arial), (A, Cloth stained with blood; B, blood stained cloth washed with detergent only; and C, blood stained cloth washed with detergent and enzyme.)

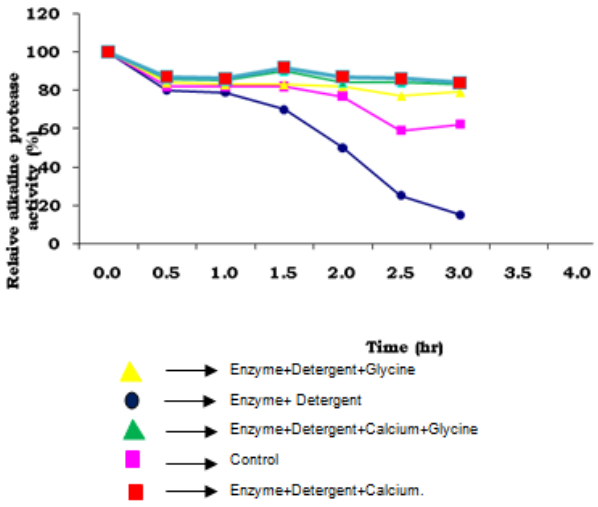


Figure 23
Capability of alkaline protease with Ariel in the presence of $CaCl_2$ and Glycine

produced in run no. 8 with X1, X2 and X3 having 28^0C, pH 9.0 and incubation period 96 h. Statistical analysis of the above data revealed that temperature has highest positive coefficient and highest SS% contribution. The process model was simulated and the observed yields of protease matched the estimated yields. From this it was inferred that it was a perfect model.

3.2. Results of Purification and Characterization of protease enzyme

The summary of purification steps involved for alkaline protease from *Streptomyces indicus* GAS-4 is reported in the Table 10.

The purification profile indicated that the enzyme was purified 15.78 fold with a yield of 44%

The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins. The molecular mass standards used were bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsinogen (24 kDa) and α– lactalbumin (14 kDa). The molecular weight was measured by interpolation from a linear semi-logarithmic plot of relative molecular mass versus the R_f value (relative mobility) (data not shown). Depending on the relative mobility, the molecular weight of the protein band was calculated to be around 30 kDa. Thus it was concluded that our alkaline protease enzyme has a molecular weight of 60 kDa. Many reports had been published on purification of different microbial proteases using ammonium sulphate precipitate and anion exchange chromatography method (Yamamato et al., 1987).The molecular weight of purified enzyme of St.halstedii Salh-12 and St.endus Salh -40 were 60 and 35 kDa. Comparative modeling predicts the 3-D structure of alkaline protease model as a given protein sequence (target) based on the template. The hypothetical 3Dstructures of template and the model are given in the above Fig. 8.

Characterization of purified enzyme

pH optimum and pH stability

For the determination of the pH optimum, phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0) and glycine-NaOH (pH 9.0-12.0) buffers were used. The highest protease activity was found to be at pH 9.0 using glycine-NaOH buffer. The results are shown in Fig.14. The stability of the purified protease was also determined by the preincubation of the enzyme in various buffers of different pH values. In the case of 2 h preincubation group, the enzyme was stable over a broad range of pH 8-10. On the other hand, in the case of 20 h preincubation group, the enzyme was stable between pH 8 and pH 9 (Fig.14).

Effect of substrate concentration on protease activity

The substrate profiles of protease activity were shown in Fig. 15 and from the graph it was observed that at 15mg concentration, protease showed the maximum velocity.

Temperature optimum and thermal stability

The activity of the purified enzyme was determined at different temperatures ranging from 30° to 90°C in the absence and presence of 10 mM $CaCl_2$. The optimum temperature recorded was at 37°C for protease activity. The enzyme activity was gradually declined at temperatures beyond 40°C. The results are shown in Fig.16. The GAS-4 protease had a half-life of 250 min. and less than 50, min at 70°C and 80°C respectively. The enzyme was almost 100% stable at 37°C even after 350 min of incubation. The results are shown in the Fig. 17.

Effect of inhibitors and chelators

Inhibition studies primarily give an insight of the nature of enzyme, its cofactor requirements and the nature of the active center (Sigma and Mooser, 1975). The effect of different inhibitors on the enzyme activity of the purified protease was studied and the results are presented in the Fig 18. Of the inhibitors tested (at 5 mM cone.), PMSF was able to inhibit the protease completely while DFP exhibited 94% inhibition. In this regard, PMSF sulphonates the essential serine residue in the active site of the protease and has been reported to result in the complete loss of enzyme activity (Gold and Fahrney, 1964). Our result were similar to that of Tsuchida et al. (1986) and Yamagata et al. (1989), who reported complete inhibition of protease by PMSF. This indicated that it is a serine alkaline protease. In the case of other inhibitors, the protease was not inhibited by EDTA, while a slight inhibition was observed with *p*CMB and β – ME.

Table 1 Effect of various variables at two levels

S.No	Name of variable	Lower level	Higher level
1	pH	6	7
2	Inoculum	3	5
3	Temperature	28^0C	32^0C
4	RPM	180	200
5	Age of inoculum	36h	48 h
6	Incubation period	48 h	96 h
7	Glucose	1%	2%
8	Yeast extract	0.5%	1%
9	Tryptone	0.5%	1%

Table 2 Placket Burmann Design plan

S.No	pH	Inoculum	temperature	RPM	Age of inoculum (h)	Incubation period (h)	Glucose	Yeast extract	Tryptone	Concentrattion unknown.
1	7	5	28	200	48	96	1.0	0.5	0.5	1362.0
2	7	3	32	200	48	48	1.0	0.5	1.0	409.0
3	6	5	32	200	36	48	1.0	1.0	0.5	301.0
4	7	5	32	180	36	48	2.0	0.5	1.0	420.0
5	7	5	28	180	36	96	1.0	1.0	1.0	1448.0
6	7	3	28	180	48	48	2.0	1.0	0.5	1157.0
7	6	3	28	200	36	96	2.0	0.5	1.0	1200.0
8	6	3	32	180	48	96	1.0	1.0	1.0	383.0
9	6	5	28	200	48	48	2.0	1.0	1.0	1064.0
10	7	3	32	200	36	96	2.0	1.0	0.5	397.0
11	6	5	32	180	48	96	2.0	0.5	0.5	340.0
12	6	3	28	180	36	48	1.0	0.5	0.5	1102.0

Table 3 Statistical analysis: ANOVA

S.No	Variable	Coefficient	SS Ratio
1	pH	66.9167	2.3586
2	Inoculum	23.9167	0.3013
3	Temperature	-423.5833	94.5055
4	RPM	-9.75	0.0501
5	Age of inoculum	-12.75	0.0856
6	Incubation period	56.4167	1.6765
7	Glucose	-35.5833	0.6669
8	Yeast extract	-6.9167	0.0252
9	Tryptone	22.0833	0.2569
10	Dummy	5.25	0.0145
11	Dummy	10.5833	0.059

Table 4 Statistical analysis: ANOVA with comments

S.No	Variable	Coefficient/effect	% SS (contribution)	Measures to be taken
1	pH	66.92	2.36	Optimization with higher pH if possible.
2	Inoculumn	23.92	0.30	Keep it as constant in optimization at higher level ie 5.
3	Temperature	-423.58	94.51	Optimization with further reduction in temperature.
4	RPM	-9.75	0.05	Keep it as constant in optimization at lower level ie180.
5	Age of inoculum	-12.75	0.09	Keep it as constant in optimization at lower level ie36 hrs.
6	Incubation period	56.42	1.68	Optimization with higher levels of incubation period.
7	Glucose	-35.58	0.67	Keep it as constant in optimization at lower level ie1.0.
8	Yeast extract	-6.92	0.03	Keep it as constant in optimization at lower level ie 0.5.
9	Tryptone	22.08	0.26	Keep it as constant in optimization at higher level ie 1.0.

Table 5 Levels of variables to be considered for experimentation

S.No	Designate	Variable	Units	Lower level	Higher level
1	X1	Temperature	C	24	28
2	X2	pH	No	7	8
3	X3	Incubation period	Hrs	48	96

Effect of metal ions

Some of the metal ions tested had slight stimulatory effect (Ca^{2+}, and Na^{+}) or slight inhibitory effect (other ions) on enzyme activity. The results are presented in the Fig.19. Addition of the metal ions Ca^{2+}, and Na^{+} increased and stabilized the protease activity of enzyme probably because of the activation of the enzyme by these metal ions. These cations also have been reported to increase the thermal stability of alkaline proteases from *Bacillus sp.* (Rahman et al., 1994; Paliwal et al., 1994). Other metal ions such as Zn^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, and EDTA did not shown any appreciable effect on enzyme activity. The residual protease activity was greater than the control when the enzyme was exposed to Ca^{++} and Na^{+} ions and same as the control when exposed to Mn^{+2}. Similar results were given by Tsuchiya K. *et al* who reported that Calcium divalent Cation raised pH and Heat stability. This is beneficial when the enzyme is used industrially.

Table 6 Factorial Design of Experimental Plan

S.No	Variables combination	X1	X2	X3	Y actual out put
		Temperature	pH	Incubation period	Enzyme activity
1	I	24	7	48	
2	X1	28	7	48	
3	X2	24	8	48	
4	X1X2	28	8	48	
5	X3	24	7	96	
6	X1X3	28	7	96	
7	X2X3	24	8	96	
8	X1X2X3	28	8	96	
9	Mid points	26	7.5	72	
10	Mid points	26	7.5	72	
11	Mid points	26	7.5	72	
12	Mid points	26	7.5	72	

Hydrolysis of protein substrates

When assayed with native proteins as substrates, the protease showed a high level of hydrolytic activity against casein and moderate hydrolysis of BSA and egg albumin.the hydrolyzing gelatin was significant and slightly lower compared to casein .The results are presented in the Fig.20. The protease showed stability and compatibility with the above commercial detergents at 37°C in the presence of $CaCl_2$ and glycine as stabilizers. As our protease showed good stability and compatibility in the presence of Ariel detergent powder. The enzyme retained more than 50% activity with most of the detergents tested even after 3 hr incubation at 37°C after the supplementation of $CaCl_2$ and glycine (Table.12). Our enzyme was found to be stable in

Table 7 Actual experimental observations with DOE plan

S.No	Variables combination	X1	X2	X3	Y actual out put
		Temperature	pH	Incubation period	Enzyme activity
1	I	24	7	48	25.4
2	X1	28	7	48	28.0
3	X2	24	8	48	18.5
4	X1X2	28	8	48	120.0
5	X3	24	7	96	16.8
6	X1X3	28	7	96	129
7	X2X3	24	8	96	20.1
8	X1X2X3	28	8	96	153.2
9	Mid points	26	7.5	72	100.0
10	Mid points	26	7.5	72	110.0
11	Mid points	26	7.5	72	104.31
12	Mid points	26	7.5	72	101.01

Table 8 Statistical analysis of the above data by ANOVA

S.No	Combination of variables	Coefficient = b	SS%
1	I	63.875	
2	X1= temperature	43.675	62.72
3	X2=pH	14.075	6.51
4	X1X2	14.975	7.37
5	X3	15.900	8.31
6	X1X3	17.650	10.24
7	X2X3	-7.200	1.70
8	X1X2X3	-9.750	3.13

Where, SS% of each variable and interactions is expressed as a percentage of individual SS to the total SS (Sum of squares) .This is also called contribution of each variable on the output. While sign of coefficient is a direction (increase or decrease), the SS % is a relative weightage of each factor over the output.

Table 9 Verification of model accuracy

Combinations	X1	X2	X3	Y actual	Y ^ estimated
	Temperature C	pH	Incubation period hrs	Enzyme concentration	Enzyme concentration
I	24	7	48	25.4	25.4
X1	28	7	48	28.0	28.0
X2	24	8	48	18.5	18.5
X1X2	28	8	48	120.0	120.0
X3	24	7	96	16.8	16.8
X1X3	28	7	96	129	129
X2X3	24	8	96	20.1	20.1
X1X2X3	28	8	96	153.2	153.2

Table 10 Summary of purification steps of alkaline protease from S.indicus GAS-4

Fraction (IU)	Total Activity (mg)	Total Protein	Specific Activity	Fold Purification	%Yield
Crude	306400	1000.2	306.4	-	100
AMM	249930	701.6	356.2	1.162	81.56
PPT					
Dialysis	200700	400.1	501.7	1.637	65.5
IEC	184048	80	2300.6	7.508	60.06
Gel Filtration	135422	28	4836.5	15.78	44.1

Table 11 Effect of substrate concentration on protease production

Substrate concentration [S]	Protease activity (IU/ml)
2	0.16
3	0.2
5	0.24
7	0.28
9	0.31
10	0.33
12	0.38
13	0.44
15	0.46
17	0.48
19	0.51
20	0.52
22	0.53

Table 12 Compatibility of alkaline protease activity with commercial detergents in the presence of $CaCl_2$ and glycine

	Relative residual alkaline protease activity (%)						
Time (h)	Control	Ariel	Mr.White	Tide	Rin	Surf Excel	Wheel
0	100	100	100	100	100	100	100
0.5	96	94	78	85	84	80	90
1.0	94	92	73	81	80	78	88
1.5	91	89	70	79	77	73	84
2.0	87	83	65	68	68	69	79
2.5	80	80	68	64	60	66	72
3.0	76	79	60	58	55	52	68
4.0	72	70	59	54	48	46	59

Guravaiah et al.
Optimization, purification and characterization of alkaline protease enzyme from *Streptomyces indicus* var.GAS-4,
Indian Journal of Engineering, 2012, 1(2), 108-117,

commercial detergents. As our protease showed good stability and compatibility in presence of ariel detergent powder, a detailed study was conducted with Ariel in the presence of 10mM $CaCl_2$ and 1M glycine for different periods (0.5 to 3 h) at 37°C. The results are shown in Fig.21. The enzyme retained about 60% activity after 1.5 hr in the presence of Ariel at 37°C and was almost inactivated after 3 hr in the absence of stabilizer. However, the addition of $CaCl_2$ (10 mM) and glycine (1M), individually and in combination, was very effective in improving the stability where it retained 50% activity even after 3 h. As the protease produced by our isolate GAS-4 was stable over a pH range of 8-10 values and also showed good compatibility with various commercial detergents tested, it can be used as an additive in detergent, to check the contribution of the enzyme in improving the washing performance of the detergent. The supplementation of the enzyme preparation in detergent i.e. Ariel could significantly improve the cleansing performance towards the blood stains. The results are shown in Fig.22.

Dehairing of animal skin with protease

The dehairing capacities of the crude and purified enzyme were studied. The ease of dehairing was noted by removing the hairs with a blunt scalpel. It was observed that the purified enzyme could dehair with greater ease than the crude enzyme. The results are shown in Fig.23.

4. CONCLUSION

The culture GAS-4 was grown in the suitable medium containing Glucose 1%, Yeast extract 0.5% and Tryptone 0.5%. The culture was shown to produce alkaline protease enzyme. Various parameters like pH, Temperature, Age of the inoculum, substrate concentration; RPM were optimized to increase the yield. A Plackett-Burman design was constructed to determine the increase in the yield and it was found to be 153.2U/ml which was most satisfactory. Further effect of various chelators, inhibitors, detergents on protease production were carried out. Dehairing activity on goat skin was performed with the isolated culture GAS-4. All the results were found to be satisfactory.

ACKNOWLEDGEMENTS

I have great pleasure in expressing my profound sense of gratitude and sincere thanks to my Guide and Teacher Dr. T.Prabhakar, Professor, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam for his invaluable suggestions, incredible patience, utmost care, keen interest and constant encouragement in shaping this research work and to bring it to completion. I express my sincere gratitude to, am immensely grateful to the Management of J.K.C College, Guntur for providing me the required infrastructure for the completion of the proposed work. I would like to thank Smt. Pavani, Smt. Jyothi and students Sirisha, Nautha, Anusha, Jahnavi, Revathi, Geetha, Rajeswari, Uma for helping me all through my project.

REFERENCES

1. Adriane, M. F. and Lacis, S. S., (1991), 'Biotechnol Lett'., vol-13 pp.- 113.
2. Akhnazarova, S. and Kafarov, V., (1982) 'In: Experimental optimization in chemistry and chemical Engineering', Moscow.
3. Auden. J., Gruner, J., Nuesch, J. and Knussel, F., (1967) 'Pathol. Microbiol', vol- 30 pp.-858.
4. Box, G. E. P. and Wilson, K. B. I., J Roy Stat Soc, (1951), vol-13, pp.-1.
5. Box, G. E. P., Hunter, W. G. and Hunter, J. S., (1978), 'Statistics for experiments' pp.- 291.
6. Chakraborty, R. and Srinivasan, M. J. Microbiol. Biotechnol., (1993), vol-8 pp.-1.
7. Cochran, W. G. and Cox, G. M. Experimental designs (1992) vol-2 pp.-335.
8. Cochran, W.G. and Cox, G. M., Experimental designs (1957), vol-2 pp.- 346.
9. Deshayes, C. M. P., Bull. Soc. Chim. Fr., (1980), vol-1, pp.-24.
10. Deshayes, C. M. P., Bull. Soc. Chim. Fr., (1980), vol-1, pp.-24.
11. Fannin, T. E., Marcos, M. D., Anderson, D. A. and Bergman, H. L., Appl. Environ. Microbiol. (1981), vol- 42 pp.-936.
12. George, S., Raju, V., Krishnan, M. R. V., Subramanian, T. V. and Jayaraman, K., Process Biochem., (1995), vol- 30 pp.-457.
13. Bhosale, S. H., Rao, M. B., Deshpande, V.V. and Srinivasan, M. C., *Enzyme Microb. Technol.,* (1995), vol- 17 pp.-136.
14. Dhandapani, R. and Vijayaragavan, R., *World J. Microbiol. Biotechnol,* (1994), vol-10 pp.- 33.
15. Donaghy, J. A. and McKay, A. M., *J. Appl. Bacterial.,* (1993), vol-74 pp.- 662.
16. Durham, D. R., Stewart, D. B. and Stelwag, E. G., *J. Bacterial.,* (1987), vol- 169 pp.-2762.
17. Fujiwara, N. and Yamamoto, *K.J. Ferment. Technol.,* (1987), vol- 65 pp.-345.
18. Gold, A. M. and Fahrney, D., *Biochem.,* (1964), vol-3 pp.- 783.

.