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Alkaline Protease production from *B. subtilis* SBS 402 isolated from Sea food processing effluent

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Keywords

Protease,
B. subtilis,
Sea food processing effluent,
Protease stability,
Dehairing activity.

Abstract

In the present study effluent samples collected from a seafood processing plant located at Chennai were plated on casein agar medium and the microbial density was found to be in the range of $3.22x10^3$ to $1.6x10^4$ CFU/g. From casein agar plate strains were screened for proteolytic activity. The most potential strain was identified as *B. subtilis*. Growth optimization for the potential strain was carried out at different pH, temperature, NaCl concentration, carbon and nitrogen sources and at different incubation time. Results showed pH 10, temperature 40°C, 2% NaCl, 1% glucose as carbon source, 0.3% peptone as nitrogen source and 24hrs of incubation were found to be optimum for the growth of the potent strain. Mass scale culture done in a shaker incubator at 150 rpm with optimized parameters resulted in an enzyme activity of 750 U/ml/min, whereas 860 U/ml/min was obtained in fermentor. Cell free extract was precipitated with 50% ammonium sulphate and after dialysis, the molecular weight of the enzyme on SDS-PAGE showed to be 28 KDa. The enzyme was found to be stable at different pH, temperature and NaCl concentration. 95 and 52% of inhibition of enzyme activity was found with inhibitors PMSF and EDTA. With 3-4 hrs of incubation of the crude enzyme (10 U/ml) with goat skin, resulted in complete removal of hair.

Introduction

Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. They are highly exploited enzymes in food, leather, detergent, pharmaceutical, diagnostics, waste management, and silver recovery. The protease enzyme constitutes two thirds of total enzymes used in various industries and this dominance in the industrial market is expected to increase in the day to come (Gupta *et al.,* 2002). Of all proteases, alkaline proteases produced by *Bacillus* species are of great importance due to their higher thermo stability and pH stability.

Bacterial proteases are easily produced in large amounts, many of them are thermostable and active at wider pH range. In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals (Gupta *et al.,* 2002). These properties make the bacterial proteases most suitable for wide industrial applications. The economic importance of proteases came to light when bacterial alkaline proteases from *Bacillus* species were introduced in 1960's to the detergent industry (Kalish, 1988).

Proteases encompass a group of enzymes, which hydrolyze peptide bonds in aqueous environment and synthesize peptide bonds in non-aqueous environment. These enzymes account for about 60% of total worldwide sales of enzymes (Rao *et al.,* I998) and alkaline proteases comprise 30% of the total worldwide enzyme production (Horikoshi, 1996).

Proteases especially the alkaline proteases are physiologically and commercially important group of enzymes used primarily as detergent additives (Banerjee *et al.,* 1999). They play a specific catalytic role in the hydrolysis of protein and are used

in industries like leather food, photography and pharmaceuticals (Moreira *et al.*, 2003).

Alkaline proteases find potential applications in the bioprocessing of used x-ray films which contains approximately 2.0 % (by weight) silver in the gelatin layers (Fujiwara *et al.*, 1991).

In recent years, the use of thermostable alkaline enzymes has increased in a wide range of other biotechnological applications, such as formulations of feed (Dharu and Sreenivasulu, 1986) and peptide synthesis (Lin *et al.*, 1996). At this juncture, effort was taken in the present investigation to isolate a potential alkaline protease producing bacterium from the effluent water of a sea food processing plant.

Materials and Methods

Isolation of protease producing bacteria

Protease producing organisms were isolated from effluent collected from a seafood processing plant located in Chennai, Tamilnadu. Samples were serially diluted using 50% aged sea water and spread plated on the surface of casein agar plates (nutrient agar with 1% casein) and incubated at 30^{o}C for 48 hrs (Naidu and Devi, 2005).

Screening for proteolytic activity

The isolated bacterial strains were purified on casein agar plates (i.e.) nutrient agar with 1% casein and they were inoculated into casein broth. After the incubation period culture filtrates were separated by centrifuging at 8000 rpm for 15min. The culture filtrate thus prepared was used for the qualitative protein production assay (Carrim *et al.*, 2006). Potential isolates were identified according to Bergey's manual of determinative bacteriology (Buchanan *et al.*, 1974) and were stored on agar slant with casein for further study.

Optimization of different parameters for maximum protease production

Different pH ranging from 7-11 (at an interval of pH 1) were maintained in the medium and incubated. Growth was assessed for every 6 hrs until 48 hrs. Likewise, different temperatures such as 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C, 45^{o}C and 50^{o}C, different salinity ranging from 0 – 3% (at the interval of 0.5%), different carbon sources (1%) such as glucose, maltose, fructose, sucrose and starch, different concentration of glucose as an ideal carbon source (0.5 – 2.5%), different nitrogen sources (0.5%) such as peptone, beef extract, gelatin, ammonium nitrate and ammonium sulphate, different concentration of yeast extract as a selected ideal nitrogen source (0.1-0.5%) were assessed for every 6 hrs until 48 hrs.

Mass scale culture

Mass scale protease production was done using *Bacillus subtlis* cells (as 1% inoculum) in the protease production medium with optimized parameters such as pH - 10, 40^{o}C, 2% NaCl, 1% glucose, 0.3% peptone was done in 2L conical flasks with 1.250L of the medium. Growth and protease activity were evaluated at the end of 24 hrs of incubation. The parameters optimized for shake flasks were used for mass scale production in a 3L lab scale fermentor. The clear supernatant was used as crude enzyme (Olajuyigbe and Ajela, 2005). Protease activity was assayed by the method of Kunitz (1947) using casein as the substrate.

Partial purification of protease

The protease from *Bacillus subtilis* was purified as per the standard protein purification procedure which involves various steps such as centrifugation, ammonium sulphate precipitation, and dialysis. 50% ammonium sulphate was used for the precipitation of the mass scale culture filtrate. The supernatant was assayed for total protein and enzyme activity.

Stability of protease

The effect of pH on protease activity was evaluated over a pH range 7-11, using different buffers such as sodium phosphate 0.1 M (pH -7), 0.1 M Tris- Cl (pH 8-10) and 0.1 M glycine NaOH (pH 11-12) in the reaction mixture. Sample was pre-incubated for 3 hrs and the enzyme activity was calculated. Similarly, temperature stability (25-50^{o}C), effect of NaCl concentration (0-3%) on protease stability were tested at 40^{o}C for 3 hrs. Enzyme activity was calculated considering the maximum activity as 100%. Regarding protease inhibitors 5 mM concentration of PMSF (Phenyl methyl sulphonyl fluoride) and EDTA (ethylene - diamine tetraacetic acid) were pre incubated with purified enzyme for 1hr. At 40^{o}C enzyme assay was done to find the extend of inhibition.

Determination of molecular weight of the enzyme by SDS-PAGE

Proteins molecular weight was determined according to the method of Laemmli (1970).

Dehairing activity

Goat skin was cut in to 5cm^{2} pieces and incubated with the crude protease enzyme in 50mM Tris-HCl (pH 8.0) at 50^{o}C.The skin was observed for removal of hair at different incubation periods.

Results and Discussion

Proteases are important industrial enzymes accounting for 60% of total global enzyme sales (Ningthoujam *et al.*, 2009). They represent one of the three largest groups of industrial enzymes (Ahmed *et al.*, 2007).

Isolation and Screening of protease producing bacteria

In the present study effluent samples collected from a seafood processing plant located at Chennai were plated on casein agar medium and the microbial density was found to be in the range of $3.22x10^3$ to $1.6x10^4$ CFU/g (Fig- 1). From casein agar plates 51 strains of varying morphology were selected and screened for proteolytic activity adopting well assay method. The zone of clearance was measured and found to be in the range of 4mm-18mm. Among the strains, the one with 18mm of zone was selected for protease production (Table 1 and Fig.2). The most potential strains was identified as *B. subtilis* by biochemical tests.

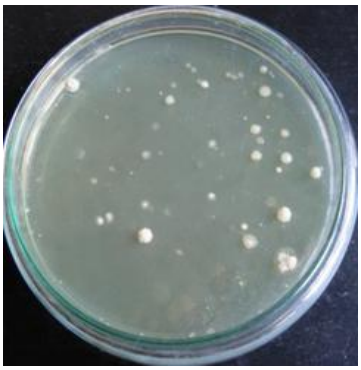


Fig. 1 - Isolation of Protease producing organism from effluent samples of seafood processing plant

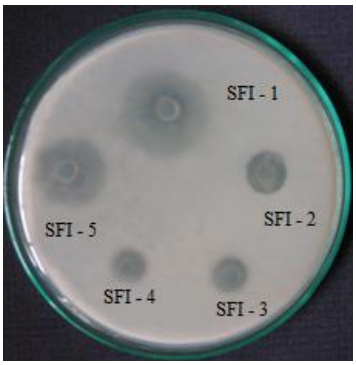


Fig.2a – Screening of the isolates (SFI 1 to SFI – 5) for protease production

Fig.2b – Isolated potent protease producing *Bacillus spp.*

Growth optimization of the potent strain

In the present study, pH 10 resulted in higher growth OD (1.232). Minimum growth of was observed at pH-7 (0.732 OD) at 24 hrs. (Fig - 3). Bajaj and Jamal (2013), found *B.pumilus* D-6 strain isolated from a dairy plant soil sample, showed protease activity at high alkaline pH (i.e) pH 8-12. In the present study also *Bacillus subtilis* was proved to be a potential source for alkaline protease production. Sharma *et al.*, 2014 also found the peak production of 362.58 U/ml at pH 8.0 in a *B. aryabhattai* K3 strain.

Regarding temperature, 40°C supported maximum growth (1.22 OD). Minimum growth (0.16 OD) was observed at 50°C (Fig-4). Similar observation was reported by Nisha and Divakaran (2014) in *B. subtilis* isolated from sea water where 40°C supported maximum protease production. Prabhavathy *et al.*, 2012 found 45°C in *Bacillus subtilis* and Gautam *et al.*, 2013 found 45°C as optimum for a Gram+ve strain producing protease.

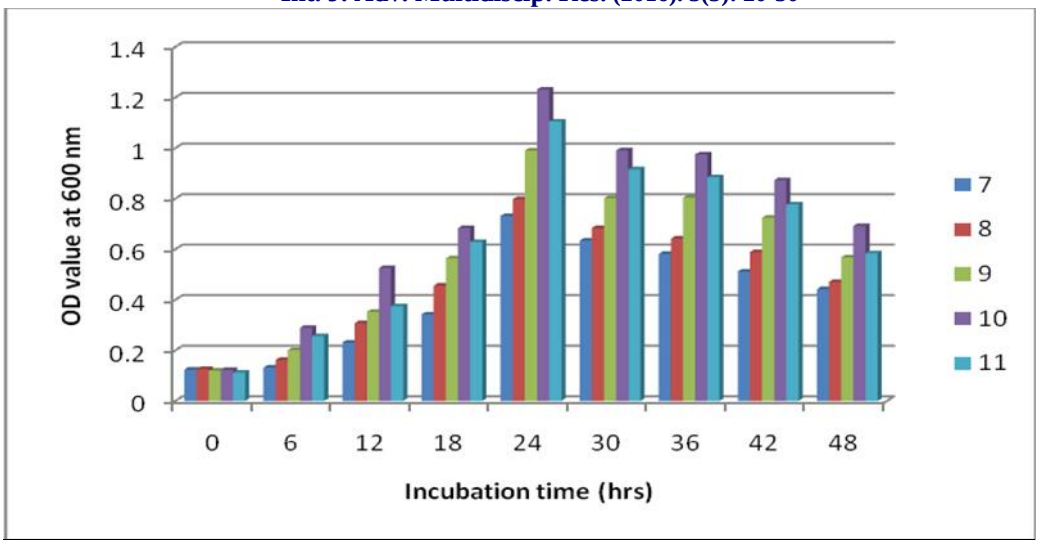


Fig. 3 – Effect of pH on growth of *B. subtilis* SBS 402

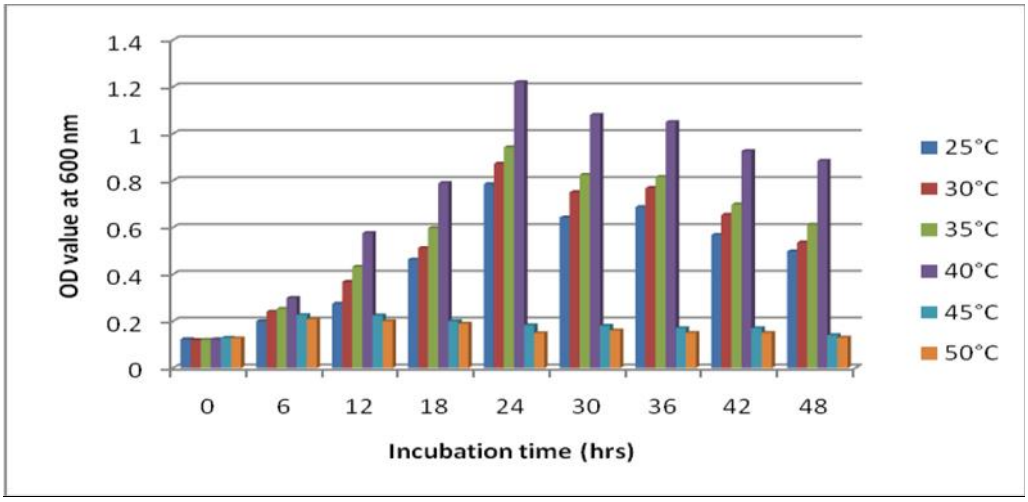


Fig. 4 – Effect of temperature on growth of *B. subtilis* SBS 402

Similarly Odu and Akujobi (2012) found 47°C as optimum for a *Bacillus* sp. When NaCl concentration of 0 to 3% were tested, at 2% NaCl maximum growth (1.09 OD) was observed and the minimum (0.53 OD) was observed at 0% NaCl (Fig. 5). Nisha and Divakaran (2014) reported that 7% NaCl favored maximum protease production in *B. subtilis*. Ravishankar *et al.*, 2012 observed a *B.subtilis* AKRS3 strain which produced an alkaline protease active at pH 9, showed the maximum growth and enzyme production at 0.01 % NaCl. Compared to these studies, the strain used in the present investigation showed the maximum growth at a higher concentration of NaCl.

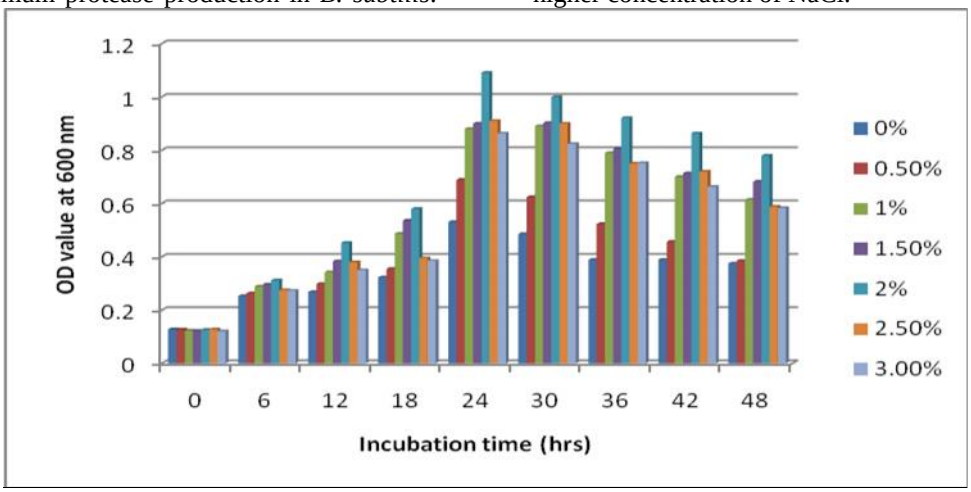


Fig. 5 – Effect of NaCl concentration on growth of *B. subtilis* SBS 402

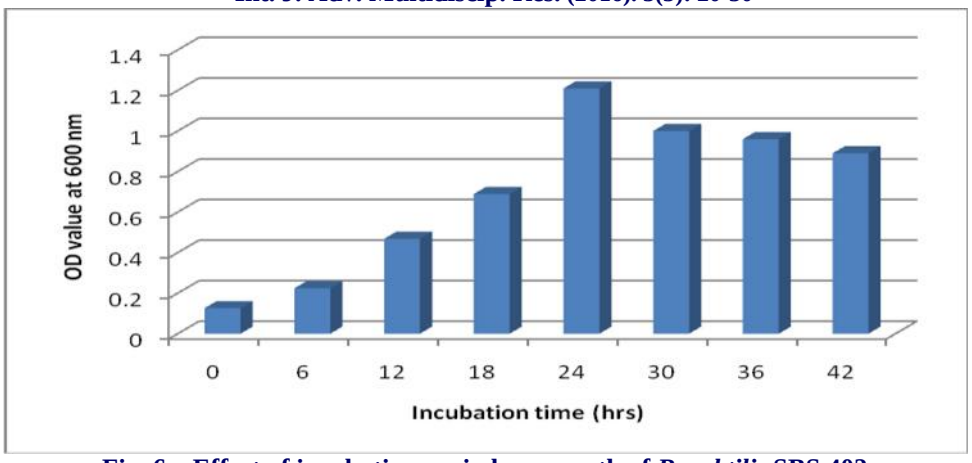


Fig. 6 – Effect of incubation period on growth of *B. subtilis* SBS 402

Among the different carbon sources tried, glucose favored the maximum growth (1.289 OD) whereas starch supported minimum growth (0.731 OD). Sharma *et al.*, 2014 reported that alkaline protease production was better with organic nitrogen sources than with the inorganic nitrogen sources and in the present study also inorganic nitrogen sources were found to be comparatively lesser supportive for alkaline protease production. In the present investigation, peptone was found to be the best nitrogen source.

Regarding concentration of glucose 1% favoured maximum growth (1.289 OD). At 2.5% of glucose the growth was reduced to 0.84 OD (Fig-7). Prasad *et al.*, 2014 found 1.0% peptone as nitrogen source, was found to be effective for maximum protease production in *B. licheniformis*. Prasad *et al.*, 2014 reported 1.5% glucose which supported maximum protease production in *Bacillus licheniformis.*

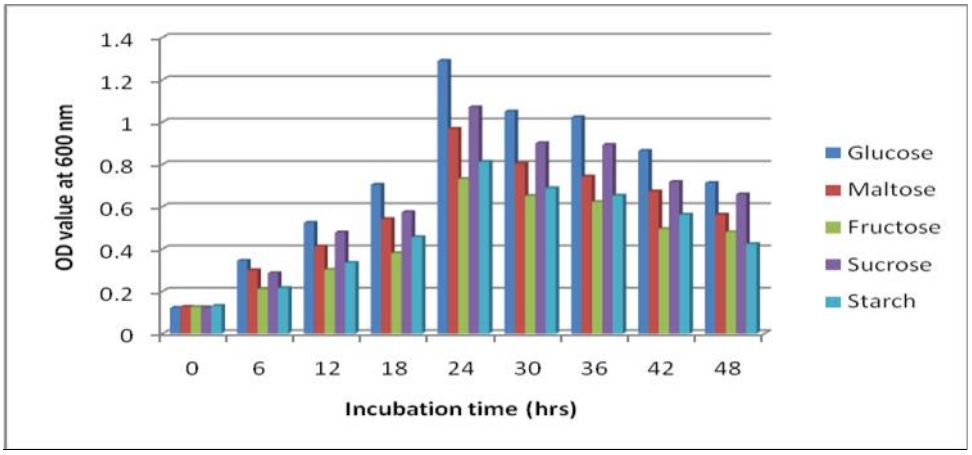


Fig. 7 – Effect of carbon source on growth of *B. subtilis* SBS 402

Peptone was used as an organic nitrogen source favoured maximum growth (0.96 OD) (Fig-8). The ideal nitrogen source concentration of 0.3% peptoned favoured maximum growth (0.96 OD) (Fig-9).

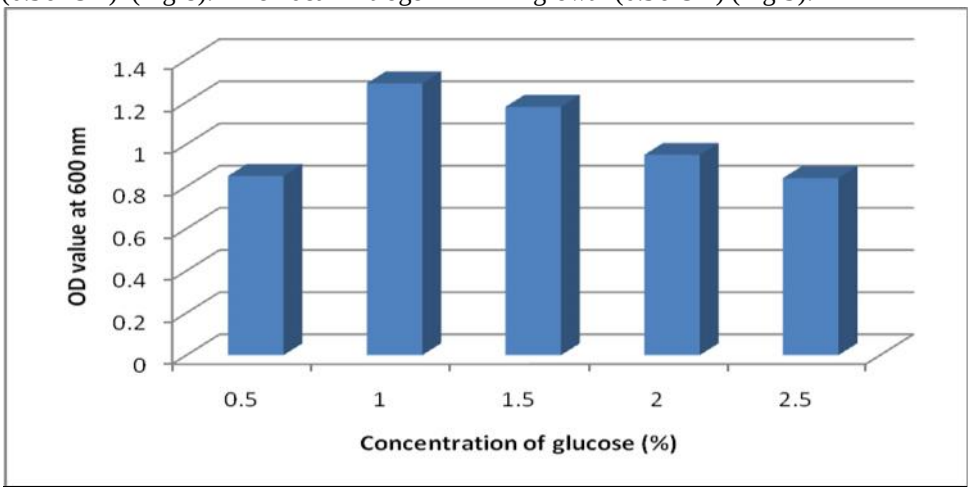


Fig. 8 – Effect of glucose concentration on growth of *B. subtilis* at 24hrs of incubation SBS 402

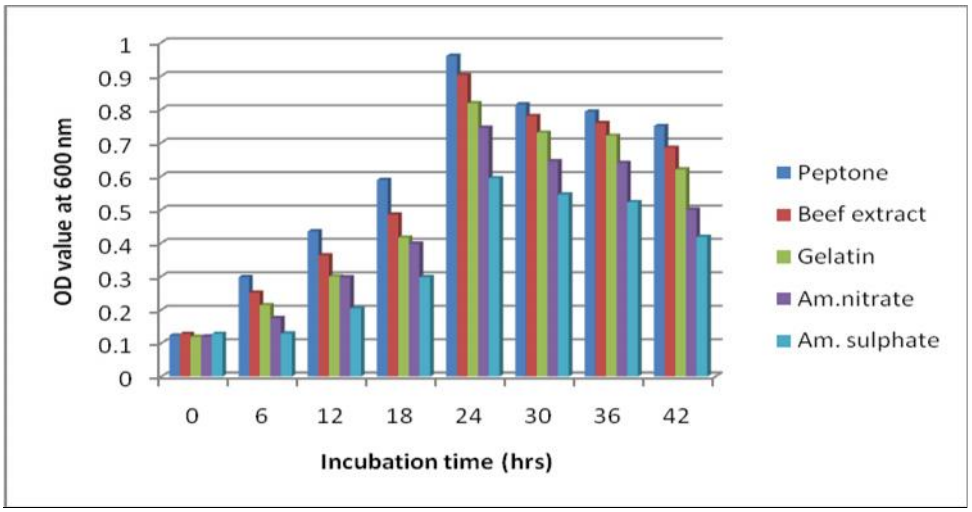


Fig. 9 – Effect of nitrogen source on growth of *B. subtilis* SBS 402

Regarding incubation period 24 hrs of incubation was found to be optimum for maximum growth (1.21 OD) (Fig.10). Jadhav *et al.*, 2013 showed that 48 hrs of incubation for *B. subtilis* was suitable time for maximum production of protease. Maximum production of proteases with 48 to 72 hrs of incubation by bacteria also reported by Hoshino *et al.*, 1997. Compared to these strains the one used in the present investigation is advantageous as it required only 24 hrs for the maximum production of the enzyme.

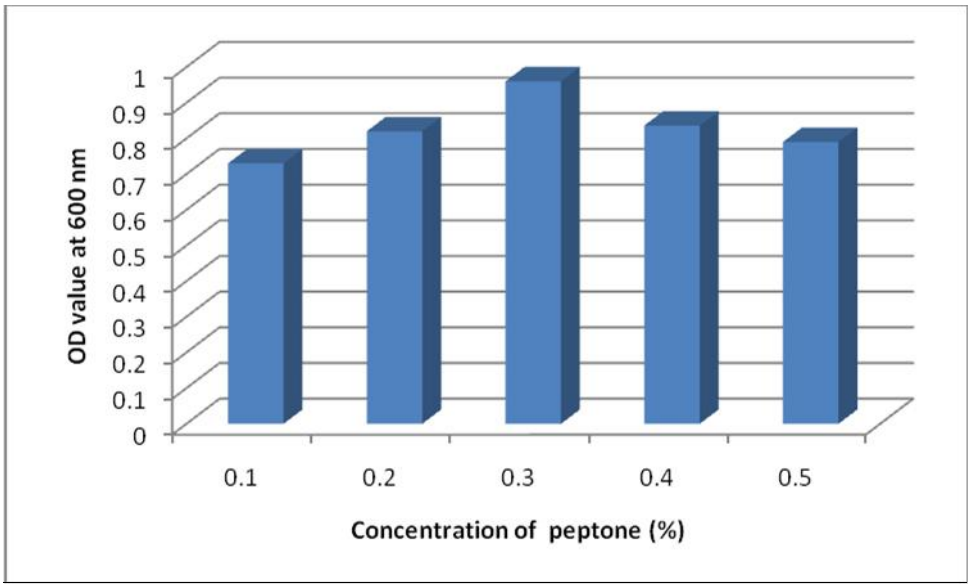


Fig. 10 – Effect of peptone concentration on growth of *B. subtilis* at 24hrs of incubation SBS 402

Mass scale culture

After optimization of all parameters separately, mass scale culture was done keeping all optimized parameters together in the medium (i.e) 40^{o}C, pH 10, 2% NaCl, 1% glucose and 0.3% peptone. Mass scale culture was done in a shaker incubator at 150 rpm. At 24 hrs, the medium with selected C, N sources resulted in an enzyme activity of 750 U/ml/min (Fig. 11 and 12). The same were followed for mass production in fermentor which exhibited an enzyme activity of 860 U/ml/min. (Fig. 13).

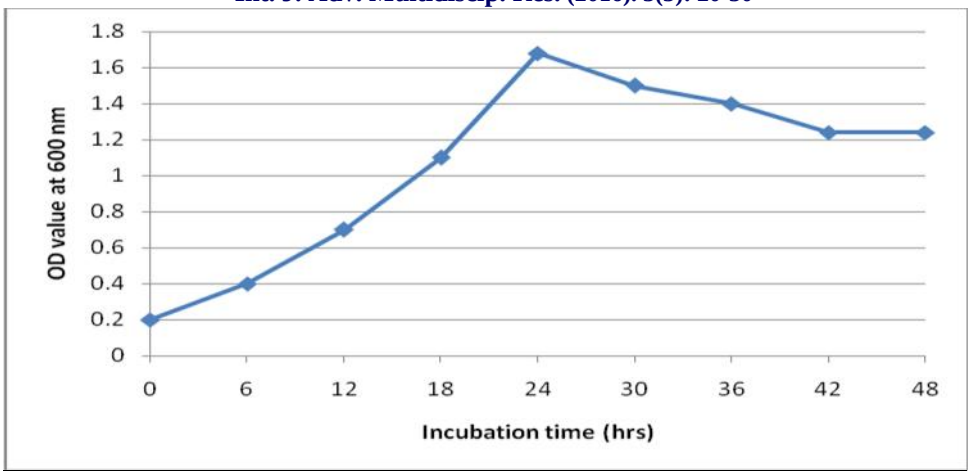


Fig. 11 – Mass scale growth of *B. subtilis* in shake flask at pH – 10, temperature 40°C, 2% NaCl, 1% glucose and 0.3% peptone SBS 402

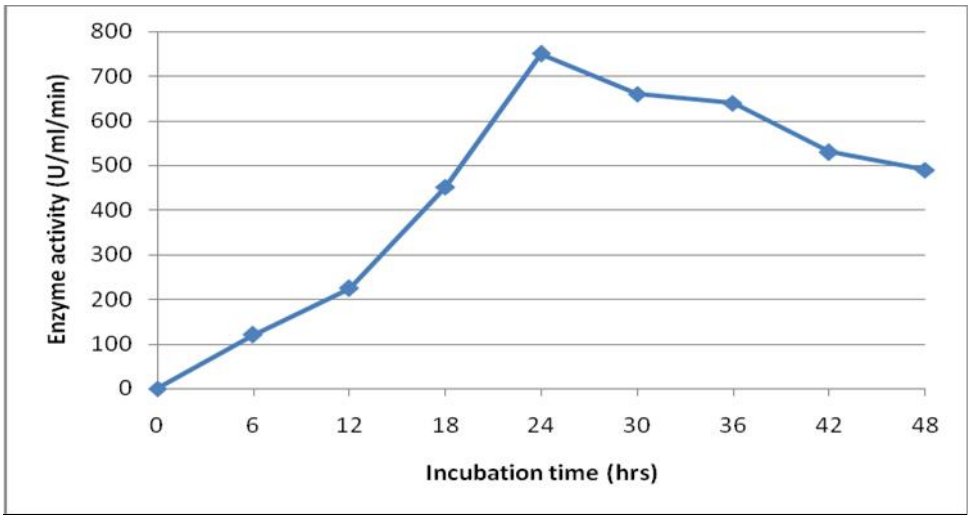


Fig. 12 – Mass scale protease production in shake flask at pH – 10, temperature 40°C, 2% NaCl, 1% glucose and 0.3% peptone using free cells of *B. subtilis* SBS 402

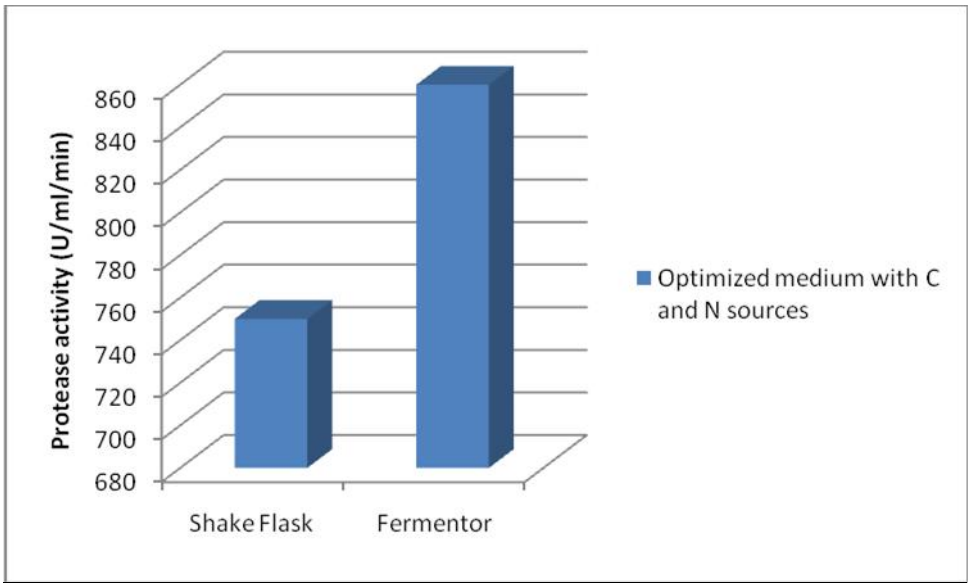


Fig. 13 – Mass scale protease production in shake flask and fermentor with free cells of *B. subtilis* SBS 402

Partial purification

Cellfree extract obtained from the mass scale culture was used for further purification. After precipitating the cell free extract with 50% ammonium sulphate and consecutive dialysis, 1ml of concentrated protease sample was assayed for protease activity and analyzed on SDS-PAGE.

Stability of protease

Metabolic activities of enzyme are very much sensitive towards pH change (Tunga *et al.*, 1998). The enzyme was found to be most active at pH 10 and moderately stable at pH 9 and 11 where it exhibited 62 and 80% activity respectively. At pH 10, 40°C and 2% NaCl enzyme activity was found to be the maximum which was considered as 100%. Respectively 46%, 58%, 82% activity was retained at pH 7, 8 and 9 where as at pH 11, 90% activity was shown.

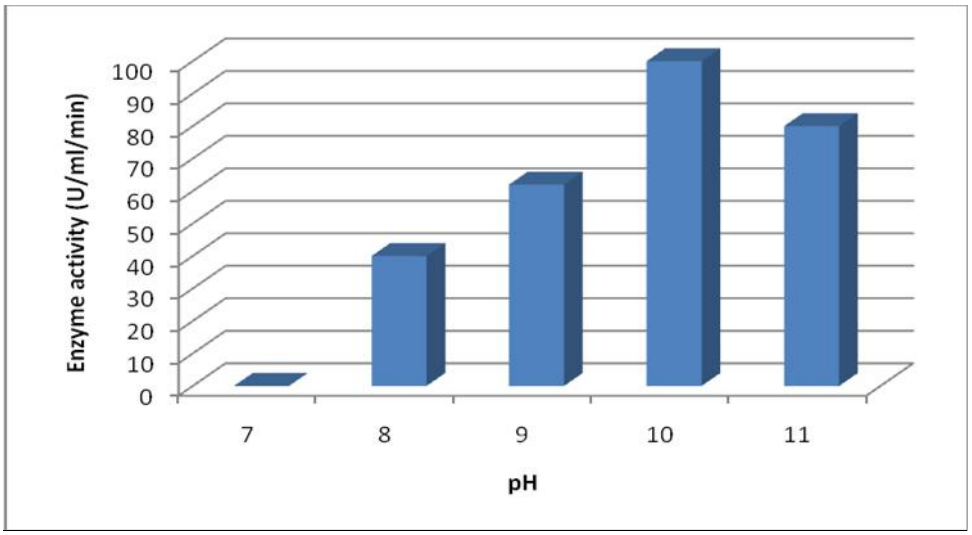


Fig. 14 – Effect of pH on protease stability produced by *B. subtilis* SBS 402

When varying temperature ranging from 25°C to 50°C at 5° interval tested at 40°C maximum activity was obtained followed by 35°C, 45°C, 50°C, 30°C and 25°C with a respective enzyme activity of 90%, 85%, 68%, 60% and 52%. In the present investigation at 35 – 45°C range, the enzyme retained around 90% of the activity. This property is of higher applied value regarding the use in detergents. Alkaline proteases showing higher activity at lower temperature are need of the hour.

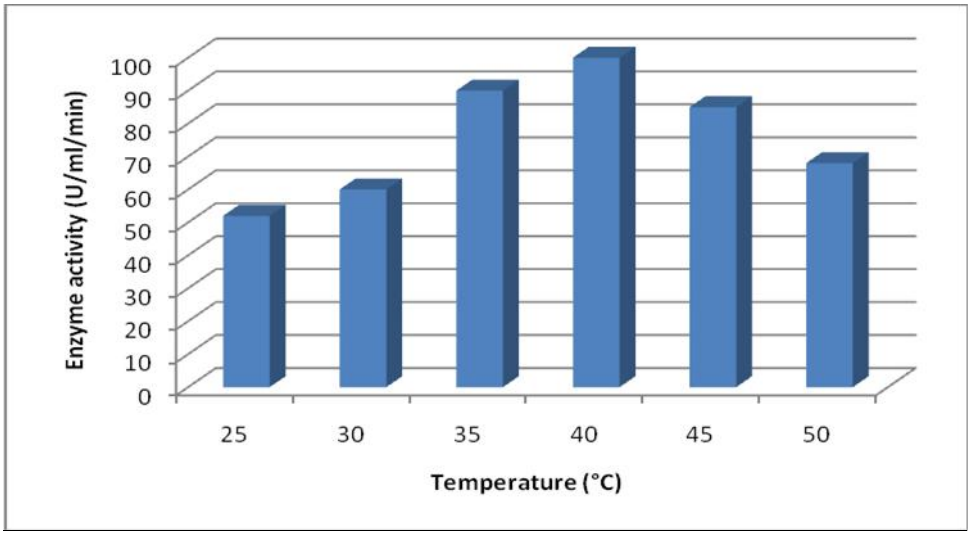


Fig. 15 – Effect of temperature on protease stability produced by *B. subtilis* SBS 402

Likewise at 2% NaCl, the partially purified enzyme showed maximum activity followed by 1.5% and 1.0% with a respective enzyme activity of 78% and 62%. Interestingly both 0.5% and 2.5% NaCl concentration showed 49% enzyme activity whereas at 0% the activity was only 25%.The study thus proved that pH 9, 40°C, and 2% NaCl were not only ideal for growth, they were ideal for enzyme production also.

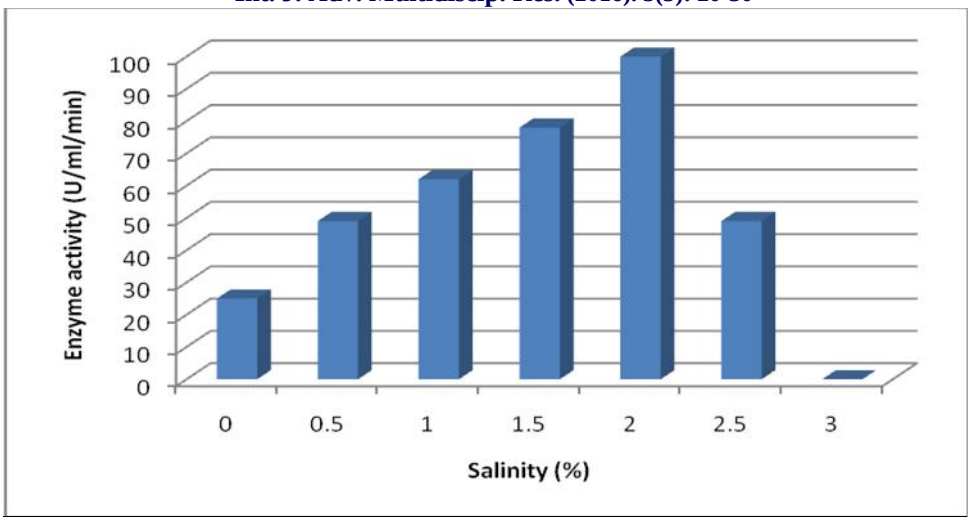


Fig. 16 – Effect of salinity on protease stability produced by *B. subtilis* SBS 402

The effects of 5mM concentration of PMSF (Phenyl methane sulfonyl fluoride) and EDTA on protease activity was studied (Fig.17). When partially purified enzyme was treated with these chemical compounds, respectively 95 and 52% of inhibition in activity was found with PMSF and EDTA. PMSF is a specific inhibitor of serine protease. As 95% of inhibition in enzyme activity was noted with this chemical compound, the protease produced by *B.subtilis* SBS 402 was confirmed as a serine alkaline protease. As only 52% inhibition was found with EDTA which is a strong metal chelator, it suggested that the partially purified enzyme may not be a metallo protease. Thus the alkaline protease enzyme was confirmed as a serine protease.

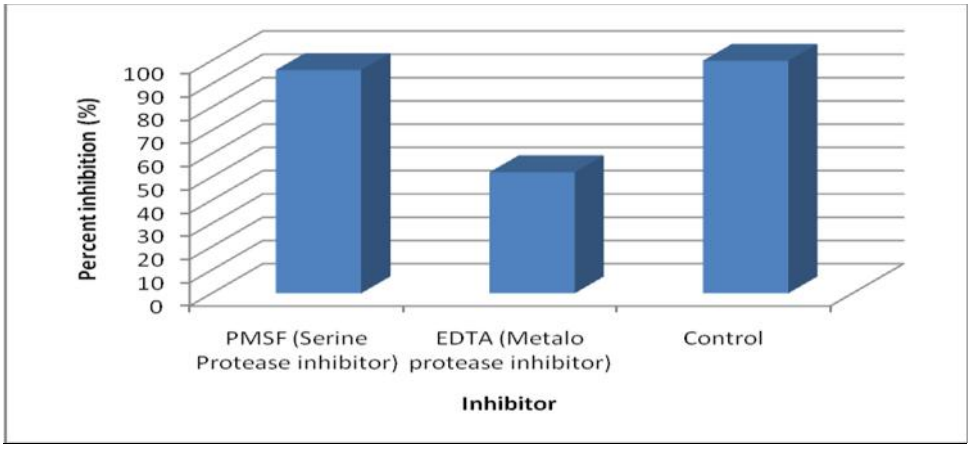
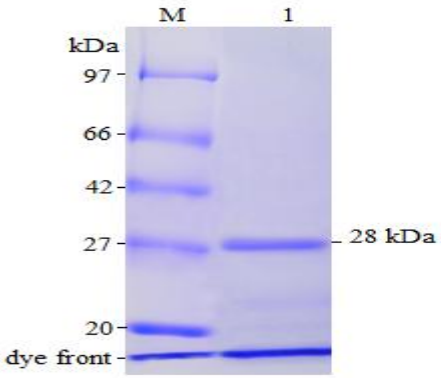


Fig. 17 – Effect of inhibitors on protease stability produced by *B. subtilis* SBS 402

The molecular weight of purified enzyme was found to be 28 KDA in SDS-PAGE (Fig-18). Prasad *et al.*, 2014 isolated protease from *Bacillus licheniformis*, analyzed on 12% SDS-PAGE and it gave a single band nearer to 52-53kDa marker protein. Khan *et al.*, 2011 reported a 29KDa protease from *Bacillus* CEMB 10370 strain.



Lane 1: Protease enzyme (28 kDa)
Lane M: Standard protein molecular weight marker

Fig 18 - Protease enzyme isolated from *B. subtilis* SBS 402 on SDS-PAGE

Dehairing activity of protease

With 3-4 hrs of incubation of the crude enzyme (10 U/ml) with goat skin, resulted in complete removal of hair (Figs.19 and 20). The protease enzyme produced by *Bacillus subtilis* SBS 402 in this study was found to be efficient in dehairing and complete hair removal was shown at 3-4hrs of incubation. Vijayaragavan *et al.*, 2014 reported crude protease isolated from *Bacillus cereus* used in dehairing process.

Fig. 19 - Goat skin incubated with enzyme and buffer with control (Left=Control, Right=Test).

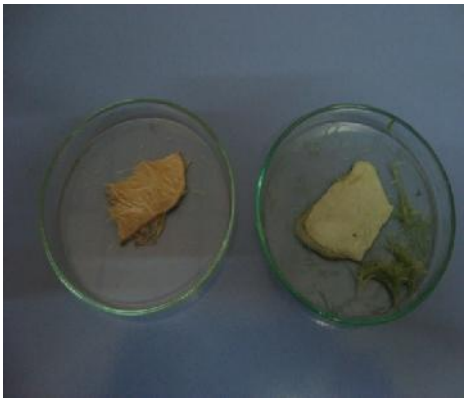


Fig. 20 - Complete hair removal after 3-4 hrs of incubation. (Left=Control, Right=Test)

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