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ENHANCED PRODUCTION OF ALKALINE PROTEASE BY *BACILLUS SUBTILIS* IN SUBMERGED FERMENTATION

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ABSTRACT

An alkaline protease producing strain was isolated from the sample collected from a slaughter house in Krishnagiri (District), T.N., India identified as *Bacillus subtilis*. Optimization of process parameters, surfactants as stimulants and strain improvement by UV for increased production of protease was carried out. Further the enzyme was partially purified and characterized. The most important factors such as temperature, pH, incubation period, carbon, nitrogen and trace elements were optimum at 35 °C, 12, 24 h, glucose, urea and $MgSO_4$, respectively. Addition of surfactants such as Tween 20 (75%), Tween 80 (25%), sodium taurocholate (35%) and SDS (55%) resulted in an increase in the yield of protease when added after 18 h of incubation of culture. On the other hand Triton X-100 inhibited the enzyme production. A 29% increase in protease production was observed by mutant strain upon UV treatment (260nm) of the parent strain for 10 min. The specific activities of the enzyme in culture supernatant and partially purified (80% saturation) samples were 0.7 and 0.6 U/mg of protein, respectively. There is an increase of 6 folds purity of protease with the recovery of 80% yield. The temperature and pH optimum of purified protease were 70 °C and 12, respectively.

KEY WORDS: **Optimization, alkaline protease, submerged fermentation, *Bacillus subtilis***

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INTRODUCTION

Proteases constitute one of the commercially important groups of extracellular microbial enzymes, accounting for about 60% of the total enzyme market [1, 2]. These enzymes have wide industrial applications, including detergent, food, pharmaceuticals, and leather, photographic and silk apart from waste treatment [3]. These enzymes also have potential to contribute in the development of high value added products due to their characteristic nature of aided digestion [4, 5]. Among all proteases, alkaline proteases are considered promising and are primarily used as detergent additive[6] and also play a specific role in the hydrolysis of protein stain such as blood, milk, human sweat etc.,. They account for about 40% of total worldwide enzyme sales and this trend is expected to increase in near future [7]. In view of this microbial strains have been exploited for the production of alkaline protease. Several microbial strains including fungi (*Aspergillus flavus, Fusarium graminarum, Penicillium griseofulvin* etc.,) and bacteria (*Bacillus licheniformis, B. firmus, B. subtilis,* etc.,) are reported to produce protease [7]. Among these, *Bacillus* genus has gained importance at industrial scale for production of proteases in submerged [8, 9, 10] and solid state fermentation [11, 12, 13].

Thermostable proteases are advantageous where processing at high temperatures can be employed, which results in faster reaction rates, increase in solubility of non gaseous reactants and products and reduce incidence of microbial contamination by mesophilic organisms. Therefore thermostable proteases are of particular interest and have become increasingly useful in the range of commercial applications [14, 15].

The aim of the present study is to investigate the alkaline protease production by *Bacillus subtilis* which was isolated and identified from the soil samples collected near to slaughter house in Krishnagiri (TN). We also report on the optimization of process parameters in submerged fermentation (SmF), including effect of surfactants and strain improvement by using UV for maximum production of the enzyme. Further the enzyme was partially purified and characterized.

MATERIALS AND METHODS

1. Isolation of strain

The bacteria isolated from a slaughter house**,** were screened for protease production on gelatin and casein agar medium, respectively [16]. The positive isolates were further screened by assaying the protease activity in liquid culture and one of the most efficient strains was isolated for further study which was characterized based on morphological and biochemical characteristic according to Bergeys manual of systemic bacteriology [17], as *Bacillus subtilis*.

2. Culture conditions

Protease production was carried out by growing in 250 ml Erlenmeyer flasks containing 20 ml of medium. The medium was sterilized by autoclaving at 121 ºC for 15 min. The medium contained the following (g/L) in distilled water: Casein, 10.0; peptone, 1.0; maltose, 4.0; $NaNO_3$, 0.5; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.02; Na_2CO_3, 1.0. The medium was inoculated and incubated at 35 °C for 24 h. At the end of stationary phase, the broth was centrifuged at 6000 rpm for 20 min at 4 ºC and the supernatant was used as extra cellular enzyme.

Enzyme assay

Protease activity was assayed by measuring the tyrosine released from the action of protease on the substrate casein.

Protease activity

Protease activity in culture supernatant was determined using spectrophotometric method [18]. Enzyme solution (1.0 ml) was incubated with 1.0 ml of 2.0% (w/v) casein in phosphate buffer (50 mM, pH 7.0) at 50 ºC for 10 min. The

undigested protein was precipitated by adding 5 ml of 5 % (w/v) Trichloroacetic acid (TCA) and allowed to stand at room temperature, followed by filtration through Whatman no.1 filter paper and 2.0 ml of filtrate was added to 4.0 ml of 0.1N NaOH and 0.5 ml of diluted folin-ciocalteau reagent. The tyrosine residues released by enzymatic hydrolysis of protein were determined [19]. A separate blank was set up to correct the non enzymatic release of tyrosine.

One unit is defined as the amount of enzyme required to release 1 µ mol of tyrosine per min under standard assay conditions.

Optimization of process parameters for increased production of protease

The procedure adopted for the optimization of process parameters influencing alkaline protease production was to evaluate the effect of an individual parameter. The parameters optimized were: (1) Incubation temperature (30-50 ºC); (2) the initial pH (5.0-12.0), the pH of the medium was adjusted by adding either 1N NaOH or 1N H_2SO_4; (3) incubation period (24-96 h); (4) carbon and nitrogen sources at a concentration of 2% (w/v), trace elements at a concentration of 0.2% (w/v) were added separately to the medium.

Surfactants as stimulants for enzyme production by Bacillus subtilis

All the surfactants were made up as stock solutions. The filter sterilized surfactants such as Tween 20 (Polyoxy ethylene sorbitan monooleate), Tween 80 (Polyethylene glycol sorbitan monooleate is a nonionic surfactant as well as an oil-in-water emulsifier), Triton X-100 (trioctyl phenoxy polyethoxyethanol), sodium taurocholate and sodium dodecyl sulphate (SDS), were added separately to a final concentration (mM) of 0.05, 0.1, 1.0, 5.0 and 10.0, respectively to one set of media prior to inoculation and incubated at 37 ºC for 24 h. In another set, the surfactants were added to the above mentioned final concentration, but after 18 hours of incubation of the culture, and again, it was incubated at 37 ºC for 24 h. Bacterial growth was determined by measuring the absorbance of the culture at 660 nm.

Mutagenesis by ultraviolet radiation

24 h old culture was taken and centrifuged at 6000 rpm and the cells were washed twice with phosphate buffer (50 mM, pH 7.0), then the pellet was suspended in phosphate buffer and transferred into sterile Petri plates. One plate was kept in dark which serves as control and rest were subjected to UV radiation (260nm) for different time intervals varying from 5 to 30 min with samples being collected at 5 min interval. After UV radiation they were kept in dark for stabilization of thymine-thymine (T-T) dimers, followed by inoculation with 0.1ml of the UV treated bacterial suspensions on gelatin agar plates. These were incubated for 48 h at 37 ºC for colony formation. After the formation of colonies, the plates were flooded with $HgCl_2$-HCl solution for 5 min and observed for transparent zones around the colonies in an opaque white background. The colonies which showed more intense zones were selected as mutants for hyper production of protease. The mutants were grown for 6 generations and protease production was tested in colonies of each generation. Those that showed better protease production than the parent strain after 6 generations were selected as stable mutants [16].

Partial purification of protease

Enzyme in the cell free supernatant portion of the culture was precipitated by ammonium sulfate (up to 80 % saturation) and incubated overnight at 4°C. The precipitate was recovered by centrifugation at 15,000 rpm for 20 min, dissolved in minimum quantity of phosphate buffer (50 mM pH 7.0) and dialyzed overnight against the same buffer. The dialyzed enzyme was tested for protease activity and for further characterization.

Effect of pH on enzyme activity

The effect of pH on the activity of enzyme was determined by holding the reaction mixtures at different pH values ranging from 5 to 12 for 30 min at 70 ºC. Buffers of 50 mM concentration

used were sodium acetate buffer (pH 4-6), sodium phosphate buffer (pH 6-8) and sodium glycine buffer (pH 8.9-12.8).

Effect of temperature on enzyme activity

The influence of temperature on the activity of protease was determined by incubating the reaction mixtures [containing 0.5 ml of appropriately diluted enzyme solution + 0.5 ml of 1% (w/v) casein in 2 ml of 50 mM phosphate buffer (pH 7.0)] at different temperatures ranging from 30 to 70 ºC for 30 min and relative enzyme activities were measured.

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RESULTS AND DISCUSSION

Enzyme yields

The organism when grown in casein broth as described in materials and methods at 35 °C for 24 h in an orbital shaker, the strain MA produced 0.7 units of enzyme per ml of culture broth.

Optimization of conditions for protease production

The enzyme production was observed from 30 to 55°C and optimum temperature for maximum yield was found at 35 °C (Fig.1).

Effect of detergent on enzyme stability

Commercially available detergents such as Wheel, Rin (Hindustan Unilever Ltd, Mumbai) and Nirma (Nirma consumer products, Ahmadabad) were dissolved at a concentration of 0.1% (w/v) respectively in distilled water, incubated with 1 ml of enzyme solution (1 U/ml) for various time intervals of 10, 20, 30, 40 and 50 min, respectively. After incubation the residual activities were recorded.

Many investigators studied the relationship between different temperatures and respective protease production and reported that protease production depends on the type of organism used, the medium conditions and type of enzyme [20]. The optimum temperature for protease [21] producing bacteria isolated from Antarctica was between 30 and 45 °C. The optimum temperatures for alkaline protease by *Bacillus* sp.MIG [22] was found to be 30 °C. Ray and Yasin [23] reported that the temperature could regulate the synthesis and secretion of extracellular proteases by microorganisms.

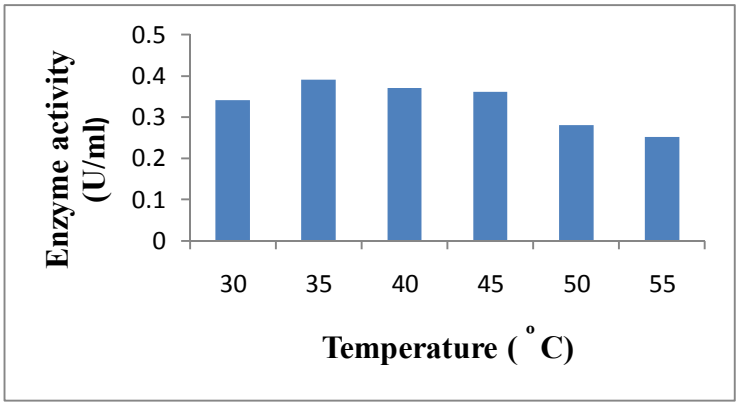

Figure. 1
Effect of temperature on protease production by B subtilis.

Alkaline protease production by microbial strain strongly depends on the extracellular pH, because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product formation [7]. In the present study results indicated that the significant enzyme

production was achieved in the pH range between 7 and 14 with maximum production of protease at pH 12 (Fig. 2). Praksham *et al.,* [5] reported an optimum pH range 9 to 11 by *Bacillus* sp. in solid state fermentation where as Sun *et al.,* [24] reported the optimal activities of protease by *Bacillus sphaericus* strain C3-41around pH 11.

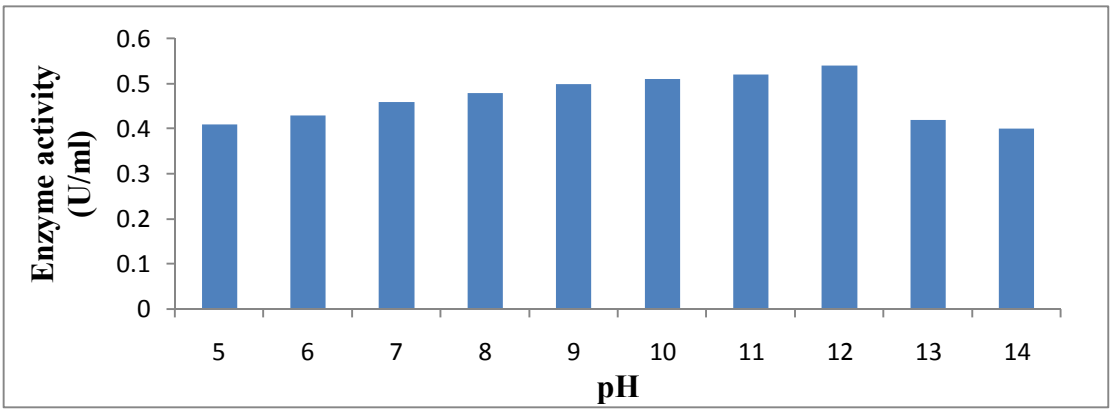

Figure. 2
Effect of pH on protease production by B. subtilis.

The result of this study showed that protease production was maximum at 24 h of incubation period (Fig. 3). The duration needed for incubation is generally dictated by the properties of the strain such as its growth rate and enzyme production pattern [25]. Thus the time requirement for product formation in the case of bacteria is obviously far less due to their faster generation time. Similar observations were recorded for protease production by *Bacillus cereus var mycoides* 3-98 and *B. anthracis* S-44 [26], whereas Elliah and Srinivasulu [27] reported maximum enzyme yield at 48 h incubation period.

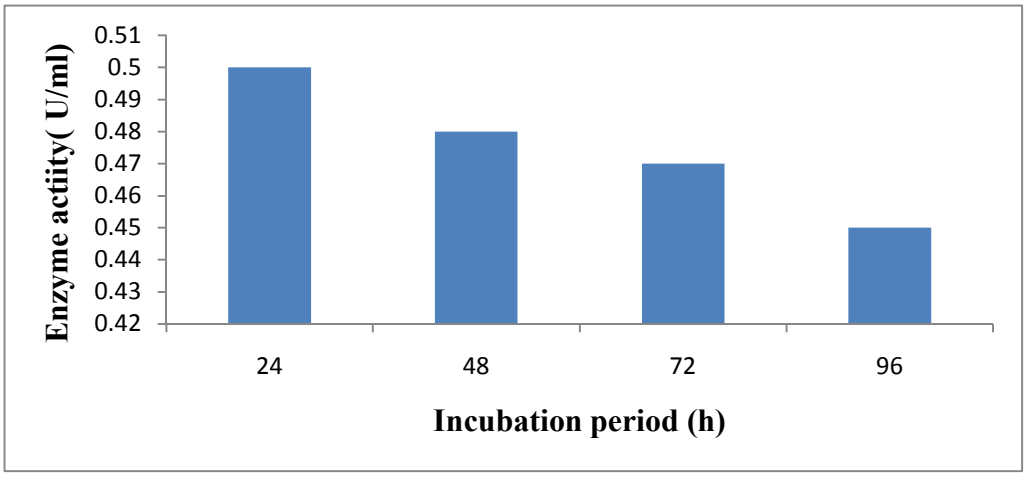

Figure. 3
Effect of incubation period on protease production by B subtilis.

Various sources of carbon such as glucose, maltose, lactose, sucrose, fructose, galactose and xylose were used to replace lactose which was the original carbon source in growth medium. Results showed that glucose was found to be the best carbon source that induced the production of protease (Fig. 4) by *B. subtilis*, when compared to other carbon

sources. This observation is in agreement with the production of alkaline protease by *Bacillus cereus* strain 146 [28]. On the other hand Yang *et al.,* [29] reported that lactose or arabinose enhanced protease production by *Bacillus subtilis*.

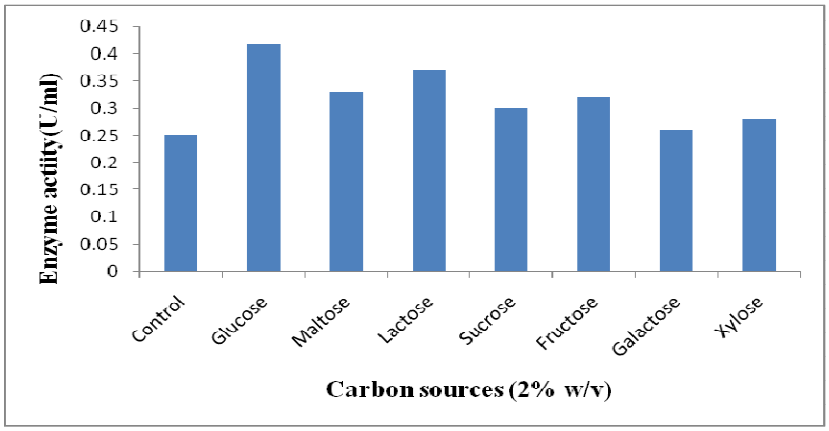

Figure. 4
Effect of carbon sources on protease production by B. subtilis.

Among the nitrogen sources tested, maximum protease production was recorded with urea (Fig. 5). Similar findings have been reported for protease production by *Bacillus licheniformis* [30]. Shafee *et al.,* [28] reported that repression of protease with the addition of inorganic nitrogen sources. Peptone have been reported as best for production of protease by *Bacillus licheniformis* [30], whereas beef extract resulted in the highest level of protease activity [28, 29]. Phadatare *et al.,* [31], reported that protease production in *Conidiobolus coronatus* was enhanced by organic nitrogen sources like yeast extract, peptone and tryptone.

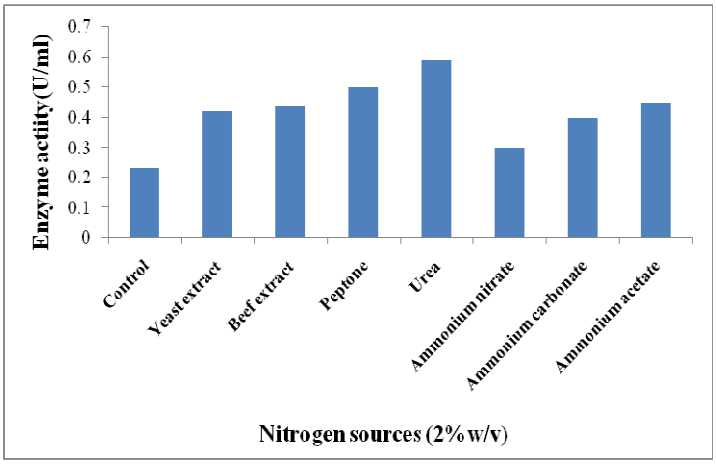

Figure. 5
Effect of nitrogen sources on protease production by B. subtilis.

Supplementation of culture medium with metal ions improved substantially the protease production of *Bacillus subtilis*. This observation strongly suggested the requirement of some metal ions for protease production by this organism. From the Fig. 6, it is clear that $MgSO_4$ yielded maximum protease when compared to $CuSO_4$, $ZnSO_4$ and $FeSO_4$.

These results are in agreement with the earlier findings which showed enhancement of protease activity in the presence of metal ions [28, 32, and 33]. It was suggested that these metal ions [34, 35] increased stability of proteases. The maximum protease activity was observed in the presence of magnesium sulphate.

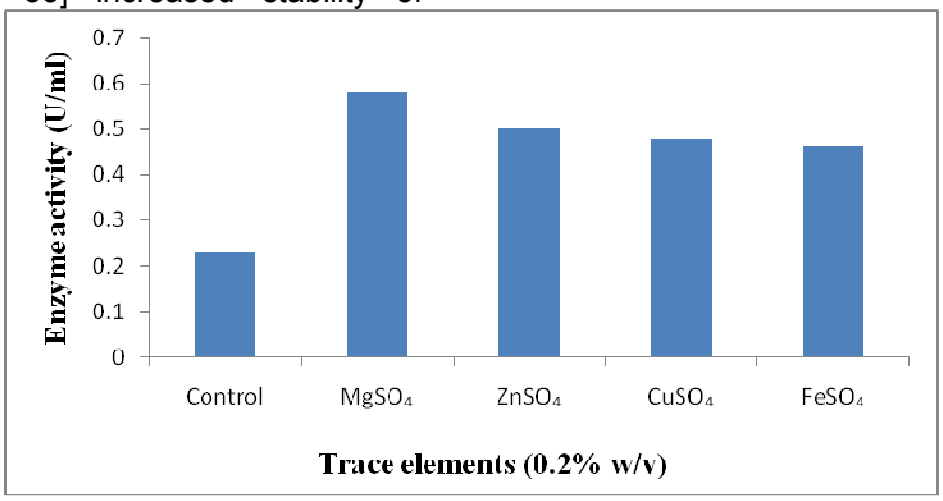

Figure. 6
Effect of trace elements (metal ions) on protease production by B. subtilis.

Addition of surfactants to *Bacillus subtilis* culture individually resulted in a marked increase in the yield of protease except triton X 100 (Fig. 7a-e). In all the cases the addition of surfactants prior to the inoculation affected the growth of organism to various extent. The stimulation of enzyme production was maximum when the surfactants were added after 18 h of incubation of culture. These findings are in agreement with the β-amylase and pullulanase production by *Clostridium thermosulfurogenes* SV2 [36]. Reese and Maguire [37] used surfactants to test seven different enzyme systems and reported that all enzymes showed increase in their productions. The enzymes used were those normally present in the extracellular production. Upon treatment with different concentration of Tween 20, Tween 80, sodium taurocholate and sodium dodecyl sulphate, *Bacillus subtilis* produced 75, 25, 35 and 55%, respectively more protease than in control.

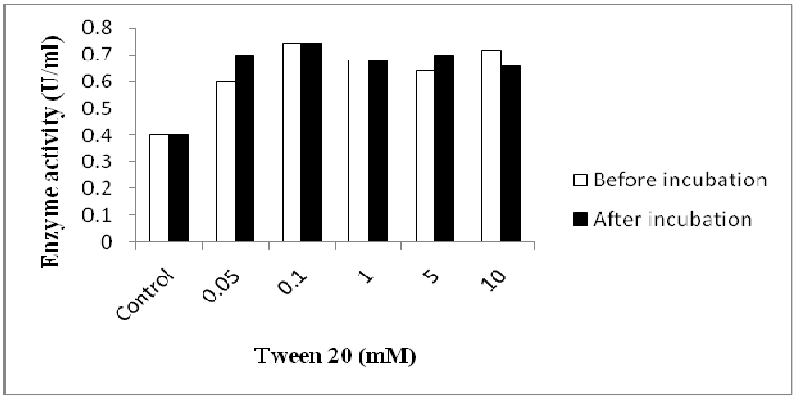

Figure.7a
Effect of different concentrations of Tween 20 on protease production by B. subtilis.

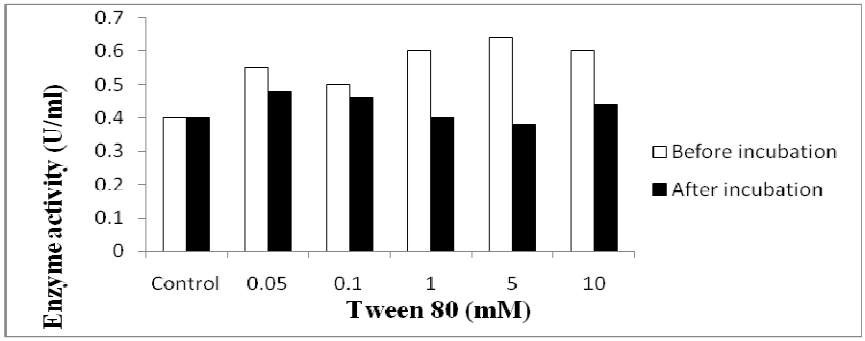

Figure 7b

Effect of different concentrations of Tween 80 on protease production by B. subtilis.

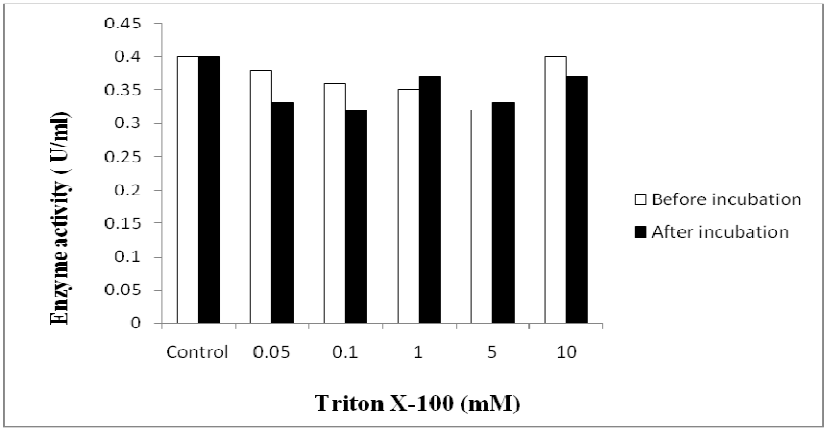

Figure. 7c

Effect of different concentrations of Triton X-100 on protease production by B. subtilis.

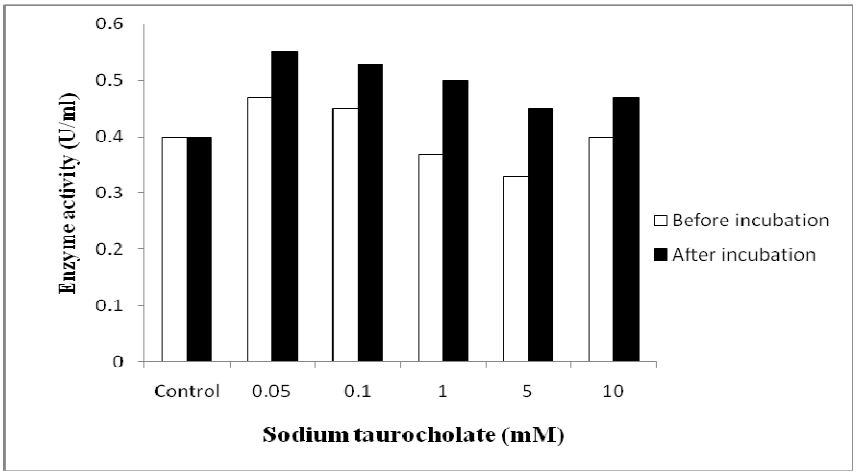

Figure. 7d

Effect of different concentrations of sodium taurocholate on protease production by B.subtilis.

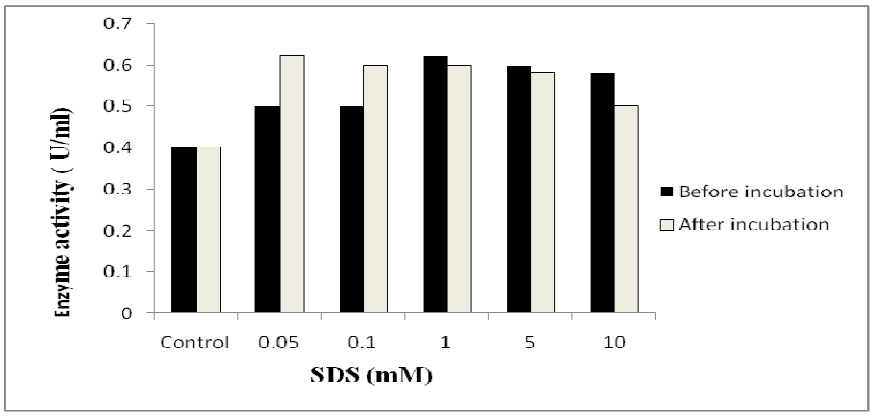

Figure.7e
Effect of different concentrations of SDS on protease production by B. subtilis.

Strain improvement by mutation

The survival data obtained on UV treatment for varying time interval are presented in Table 1. Studies on protease production from *Bacillus sp* [10, 20] as well as on mutation of *Pseudomonas* sp. for protease production have been reported. There are no reports available in the literature on ultra violet (UV) mutation of *Bacillus subtilis* for protease production. In the present study mutated strains showed 28% more protease production than the parental strain, whereas *Pseudomonas* mutant showed 2.5 fold increases in protease production over the parental strain [10].

Table: 1
Survival data for Bacillus subtilis MA on UV treatment

UV light exposure (min)	Number of colonies germinated	Survival (%)
0	2000	100
5	18	0.40
10	15	0.31
15	10	0.20
20	8	0.18
25	3	0.05
30	1	0.02

Survival (%) was calculated as follows
For a treatment time (t) survival (%) = 100* (colony count obtained for time (t)/2000)

Partial purification and characterization of protease

The enzyme produced by the strain MA was partially purified by ammonium sulphate precipitation. The strain produced 769 units of thermostable protease per litre of culture broth. After salting out with ammonium sulphate, the partially purified crude sample contained approximately, 540 units of protease per litre. In the present study the specific activities of protease in culture supernatant and partially purified samples were 0.71and 0.6 U/mg protein, respectively. There is an increase of 6 folds of purity of protease with the recovery of 80% (Table 2)

Table: 2
Summary of partial purification procedure of Bacillus subtilis.

Procedure	Enzyme yield U/litre	Total protein (mg)	Specific activity (U/mg rotein)	Purification factor	Yield (%)
Culture supernatant	769	549	0.71	1	100
Salting out with NH_4SO_4 (80%) and dialysis	540	379	0.60	6	80

Effect of pH on enzyme activity

The optimum pH for protease activity was found in the range of 12 to 13 (Fig. 8). The enzyme was active in alkaline conditions, indicating its potential use in detergent formulation as reported by Gupta *et al.,* [6], and stated that alkaline protease which was useful for detergent applications are mostly active in pH range 8 to 12. Al-Shehri [30] reported that an optimum pH of 9 was found for activity of protease.

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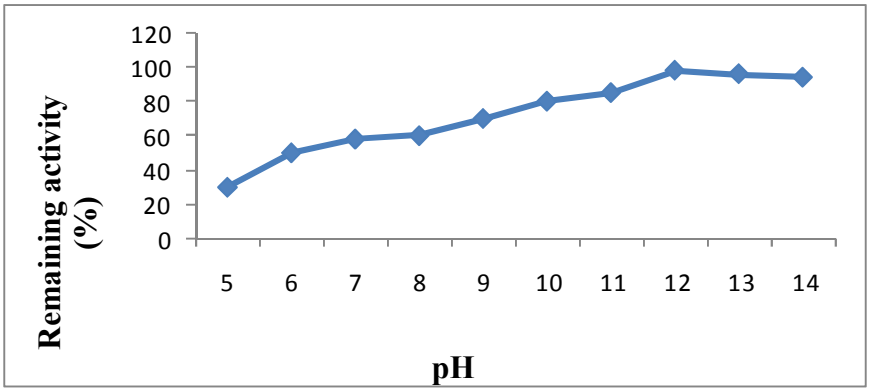

Figure .8
Effect of pH on protease activity

Effect of temperature on enzyme activity

The optimum temperature for the protease activity by strain was 60 °C (Fig. 9) which was similar to that described for other *Bacillus* proteases [14, 15].

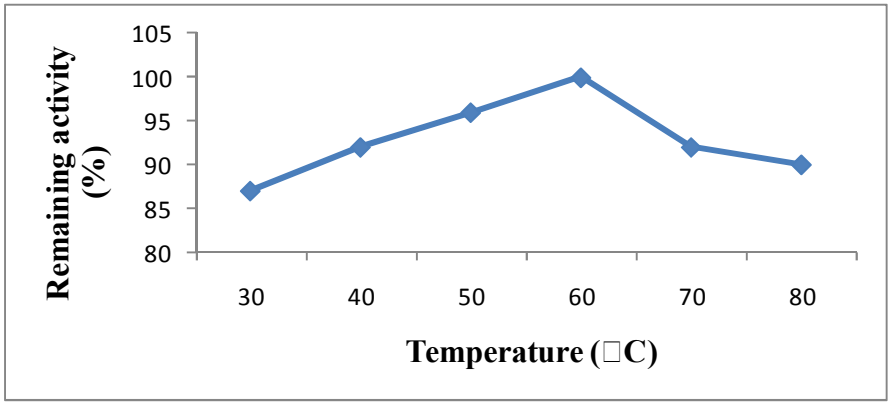

Figure. 9
Effect of temperature on protease activity

Detergent stability

Among the three detergents (Nirma, Wheel and Rin) tested for stability on protease, wheel resulted in better stability on protease (Fig.10).

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Rahman *et al.,* [34] reported that SSR1 protease retained 40 to 90 % of its activity in the presence of local detergents. This property of the enzyme is very useful for application in detergent industries.

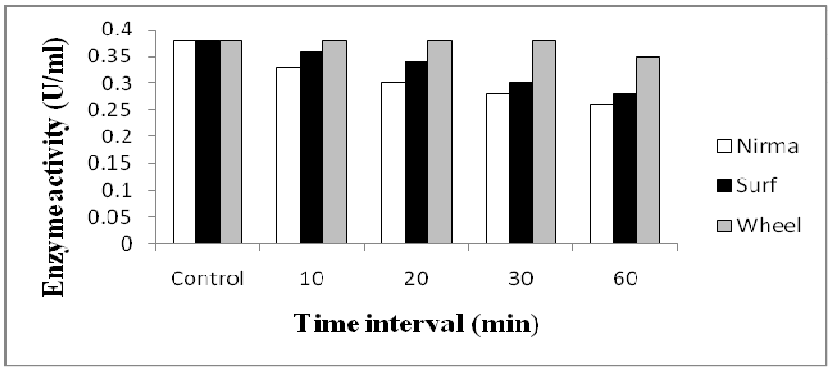

Figure. 10
Effect of detergents on protease stability

CONCLUSION

The results of the present study showed that the yields of protease were increased under optimized parameters with a medium containing lactose as carbon source. The enzyme yields were 8.6 times more than before optimization. Therefore these results, clearly indicated, that the strain *Bacillus subtilis* MA is considered more promising for the production of extracellular protease enzymes. In conclusion, the ability of *Bacillus subtilis* protease enzyme to withstand a high temperature (of up to 70 °C for 2 hours), alkaline pH and high stability in the presence of detergent suggests that the enzyme has a potential application in detergent industries

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