Enhanced production of protease from *Bacillus subtilis* AP-CMST W3 and its application in leather processing and silver recovery

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ABSTRACT

In the present study, *Bacillus subtilis* AP-CMST W3 was isolated from an estuarine environment for the production of protease for various biotechnological applications. The process parameters were optimized to enhance the production of protease by the candidate organism. Protease production was found to be optimum in the presence of 0.5% maltose, 0.5% casein, 0.1% magnesium chloride, 3% sodium chloride, at pH 7.0 and 40 °C. The enzyme was purified by the combination of ammonium sulphate precipitation, dialysis and sephadex G-75 gel filtration chromatography. The molecular weight of the purified enzyme was estimated as 21 kDa. The purified enzyme demonstrated appreciable activity at higher temperature (40 °C) and pH 7.0. The enzyme showed potent activity on various surfactants and detergents. It dehaired goat skin and hydrolyzed the gelatine layer of used "X" ray film. These properties reinforce the possibility of inclusion of protease from *B. subtilis* AP-CMST W3 in leather processing industry.

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Key words: protease, optimization, dehairing, silver recovery.

1 Introduction

Microorganisms are important sources to produce enzymes for industrial applications. Proteases are the group of extracellular enzymes and account for 60% of the total enzyme sales worldwide (Banerjee *et al.*, 1999). These enzymes find greater applications in feather processing, silk degumming, bioremediation and pharmaceuticals. Among proteases, alkaline proteases have major application in food processing, waste water treatment and peptide synthesis (Gupta *et al.*, 2002). In recent years, proteases from *Bacillus* spp. have been exploited for their dehairing activity (Soundararajan *et al.*, 2011), organic solvent-stability (Rai and Muckerjee, 2010), feather degrading (Rai and Muckerjee, 2011), detergent stability (Haddar *et al.*, 2011) and silver recovery process (Vijay *et al.*, 2010).

Proteases are obtained from various organisms such as animals, plants, archaea and bacteria (Sevinc and Demirkan, 2011). Among various sources, proteases from microbial origin are preferred because production of enzyme with desired application through gentic manipulation (Josephine *et al.*, 2012). A wide range of bacteria are known to secrete proteases and a large proportion of these enzymes were exploited from the genus *Bacillus*, because of their potential industrial applications. Proteases have been characterized from various *Bacillus* sp. including, *B. amyloliquefaciens* (George *et al.*, 1995), *B. subtilis* 11QB32 (Varela *et al.*, 1997), *B. circulans* (Subba Rao *et al.*, 2009), *B. licheniformis* NCIM2042 (Bhunia and Dey, 2012), *B. licheniformis* P003 (Sarker *et al.*, 2013) and *B. caseinolyticus* (Mothe and Sultanpuram, 2016). Studies showed that physical factors such as pH (Tobe *et al.*, 2005), incubation time (Yossan *et al.*, 2006), and nutritional factors such as carbon and nitrogen sources significantly influenced on protease production by bacteria (Prakasham *et al.*, 2006). Considering the significance of proteases from *Bacillus* sp. an attempt has been made to optimize the protease production from *Bacillus subtilis* AP-CMST W3 for various biotechnological applications.

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International Journal of Advancements in Research & Technology, Volume 5, Issue 6, June-2016
ISSN 2278-7763

2 MATERIALS AND METHODS

2.1 Screening and identification of proteolytic bacteria

Water sample was collected in a sterile container from Rajakkamangalam estuary, Kanyakumari District, Tamilnadu India. Collected sample was serially diluted upto $10^{-7}$and the dilutions were poured on skimmed milk agar plates supplemented with 2.5% sodium chloride and 1% skimmed milk. The plates were incubated at 37 °C and a potent protease producing bacterial isolate was selected based on the zone of clearance on skimmed milk agar plates. AP- CMST W3 was observed as a strong protease producer in skimmed milk agar plates. This protease producing bacterial isolate was identified through biochemical tests and 16S rRNA gene sequencing. Genomic DNA was extracted and it was amplified by using the forward primer (5′ GAGTTTGATCCTGGCTCAG3′) and a reverse primer (5′ ACGGCTACCTTGTTACGACTT3′). The amplified PCR product was sequenced and the 16S rRNA gene sequence was identified as *Bacillus subtilis* APCMST W3 and it was submitted to Genbank under the accession number KF009681. This potential protease producing strain was further selected for optimization studies to enhance the protease production.

2.2 Assay of proteolytic activity

Protease activity was determined by a modified procedure based on the method of Takami *et al*. (1989). The 18- h old culture of *B. subtilis* AP-CMST W3 was innoculated into the nutrient broth containing 2.5% NaCl, 1% casein at pH 7.0 and kept in a shaker incubator for 48 h (150 rpm) at 37 ºC. After 48 h, the culture was centrifuged at 10,000 rpm for 15 min at 4 °C and the cell free supernatant was used for protease assay. In brief, 0.5 ml of culture supernatant was mixed with 1.0 ml casein (pH 8.0, 100 mM) and incubated at 37 °C for 30 min. The enzyme reaction was terminated by adding 2.5 ml TCA (110 mM) and incubated at room temperature for 30 min. Further, 2.5 ml of Na_2CO_3 (500 mM) was added, and incubated for 30 min at 37 °C. To this mixture 0.5 ml of Folin - Ciocalteus reagent was added and the absorbance was read at 660 nm using a UV-Visible spectrophotometer. The amount of protease produced was calculated using tyrosine standard graph. One unit of protease activity is defined as the amount of enzyme required to liberate 1 μmol of tyrosine per min under the standard assay condition. The range of concentration (10–100 μg) of tyrosine was used as standard. All the experiments were carried out in triplicates and average values were recorded.

2.3 Total protein estimation

The total protein content of the supernatant was determined as described by Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) was a standard.

2.4 Effect of nutritional factors on protease production

In the present study, the protease production by *B. subtilis* APCMST-W3 was optimized through supplying various nutrient sources such as carbon, nitrogen, and metal ions individually in the basal medium. The influence of carbon sources (0.5%, w/v) on enzyme production was studied by using five different carbon sources such as maltose, lactose, sucrose, fructose and galactose. To study the influence of nitrogen sources (0.5%, w/v) on enzyme production, five different nitrogen sources such as casein, skimmed milk, urea, ammonium sulphate and ammonium chloride were supplemented. Effect of ionic sources on enzyme production was studied by supplementing metal ions such as magnesium chloride, barium chloride, copper sulphate, calcium chloride, zinc sulphate, zinc chloride, mercuric chloride and EDTA at 0.1% (w/v) level with the basal medium.

2.5 Effect of environmental factors on protease production

Effect of sodium chloride on protease production was evaluated by supplementing sodium chloride at various concentrations (1, 2, 3, 4, 5, 6 and 7%, w/v) in the basal medium. Effect of pH on enzyme production by the candidate organism was studied by adjusting the pH of the culture medium with various pHs such as 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. Effect of temperature on protease production was studied by incubating the basal medium inoculated with *B. subtilis* AP-CMST W3 at various temperatures (10, 20, 30, 40, 50, 60 and 70 °C) over a period of 72 h. After 72 h of incubation, the enzyme was extracted and the protease activity was assayed.

2.6 Purification of protease from *B. subtilis* AP-CMST W3

The protease was purified by the combination of ammonium sulphate precipitation, dialysis and sephadex G-75 gel filtration chromatography techniques. Solid ammonium sulphate was added slowly to the

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ISSN 2278-7763

crude enzyme with constant stirring till attaining 40-80% saturation. The pellet was recovered by centrifugation at 10,000 rpm for 10 min. Then the pellet was resuspended in 2.0 ml double distilled water and dialyzed overnight against water (two changes), and sodium phosphate buffer (pH 7.4, 100 mM) (third change). The dialysed fraction was loaded on sephadex G-75 gel filtration column and was eluted using the same buffer as a mobile phase. Twenty five fractions were collected in clean test tubes under ice cold condition to avoid enzyme denaturation. The extinction was measured at 280 nm using a UV- Visible spectrophotometer and enzyme activity was assayed in all fractions.

2.7 SDS-PAGE and zymography analysis

SDS – PAGE was prepared according to the method of Laemmli (1970). 20 µl of purified protein was mixed with 20 µl sample buffer (10% glycerol, 2% SDS, 62.5 mM Tris-HCl buffer) containing β – mercaptoethanol. Molecular weight was estimated by using myosin rabbit muscle, phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase as standards. To determine protease activity on the gel, 11% polyacrylamide gel was co-polymerized with 1.0% casein. The sample was not boiled at 100 Cº for 1 min and was not denatured with β-mercaptoethanol, and run at constant voltage (50 V). The gel was stained with coomassie brilliant blue followed by destaining. The protease enzyme appeared as a clear zone on the gel.

2.8 Effect of sodium chloride, pH and temperature on enzyme activity

The effect of sodium chloride on enzyme activity was studied by incubating 0.1 ml of purified protease with various concentrations of sodium chloride (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3% NaCl) at 37 °C for 30 min. The effect of pH on enzyme activity was determined by the reaction of 0.1 ml of purified enzyme with 1 ml of casein substrate (1%, w/v) in various pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) at 37 °C for 30 min. The effect of temperature on enzyme activity was studied by holding reaction between purified enzyme along with 1 ml of casein substrate (1%, w/v) at various temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C) for 30 min. The impact of inorganic ions on enzyme activity was determined by substituting the individidal purified enzyme with different metal ions such as barium, zinc, magnesium, manganese, mercury, and copper at 10 mM concentration.

2.9 Effect of surfactants and detergents on enzyme stability

Stability of protease towards various surfactants such as sodium dodecyl sulphate (SDS), Tween 20, Tween 40, Tween 60, Tween 80, Triton X 100 and polyethylene glycol (PEG) was evaluated individually with 1% (v/v) level each. The stability of obtained protease towards various detergents was also studied. The detergents used were: Henko, Ujala, Tide+, Ariel, Sunlight, Mr. White and Surf excel. For these, the surfactant and detergent solutions were prepared and mixed with 0.1 ml of purified enzyme produced by *B. subtilis* AP-CMST W3 was added and it was incubated for 1 h at room temperature (30 ± 1 °C). The enzyme activity of a control sample (without surfactant/detergent) was taken as 100%.

2.10 De-hairing properties of protease

The de-hairing property of the enzyme was carried out at room temperature with crude enzyme preparation from an industrial point of view. Goat skin was cut into small pieces (2 × 2 cm) and incubated with purified enzyme at a final concentration of 250 U/ml for up to 24 h. The control skin was incubated in solution devoid of enzyme. The skin pieces were then observed for dehairing nature (Huang *et al.,* 2003).

2.11 Effect of enzyme on silver recovery

Used X-ray film was used to study the effect of protease produced by the bacterial isolate *B. subtilis* AP-CMST W3 on silver recovery. X-ray film was washed with double-distilled water and impregnated with ethanol. The washed X-ray film was dried in an oven at 40 °C for 30 min. About 0.5 g of X-ray film (2 cm · 2 cm) was incubated in 10 ml purified enzyme (250 U/ml) at room temperature (28.0 ± 1 °C) for 24 h. After 24 h, turbidity was observed because of silver ions removed from the gelatin layer of used X-ray film.

3 RESULTS AND DISCUSSION

Each microbial isolate is unique in their metabolic, biochemical and molecular and enzyme production properties. This warrants optimization of culture conditions to enhance the production of enzymes from any new bacterial isolate. In the present study a potent

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ISSN 2278-7763

Table 1. Morphological and biochemical characters of *B. subtilis* AP-CMST W3.

Experiment	Result
Gram's staining	+ve
Shape	Rod
MR	+ve
Urea	+ve
Starch	+ve
Motility	+ve
Glucose	-ve
Lactose	-ve
Sucrose	+ve
H2S	-ve
Casein	+ve
Indole	-ve
Citrate	-ve
Oxidase	-ve
Catalase	-ve

bacterial isolate, which could able to produce protease was isolated from an estuarine environment. Initially eight protease positive isolates were screened. Among these only one strain was identified as potent strain and it produced maximum amount of protease which showed maximum zone of hydrolysis (9 mm) on skimmed milk agar plate when compared to other isolates. The morphological and biochemical characteristics of this strain was found to be rod shaped bacterium and showed NaCl tolerance upto 2.5%. It's morphological and biochemical characteristics are presented in Table 1. Based on 16S rRNA analysis, it was

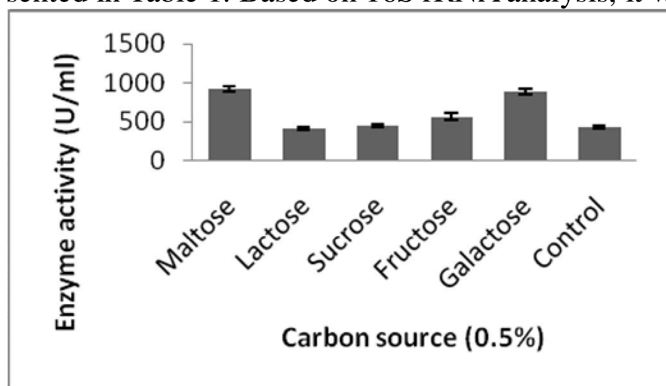

Fig. 1a. Effect of carbon sources on enzyme production by *B. subtilis* AP-CMST W3

identified using BLAST (NCBI) search; it displayed 100% sequence similarity with *Bacillus subtilis*. Therefore it was identified as *B. subtilis* APCMST W3.

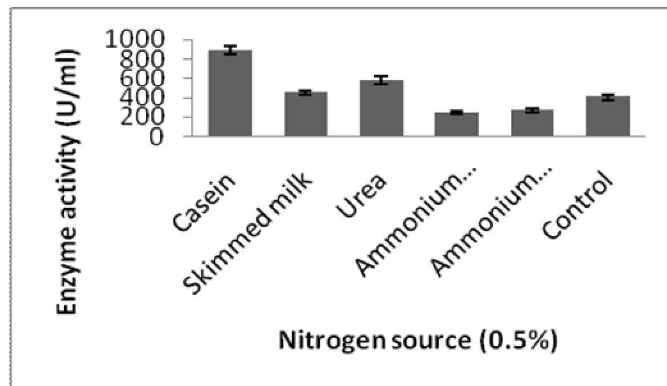

Fig. 1b. Effect of nitrogen sources on enzyme production by *B. subtilis* AP-CMST W3

The effect of carbon sources on enzyme production by the candidate organism is shown in Fig 1a. Maximum (919.7 ± 3.33 U/ml). protease production was obtained in the medium containing 0.5% maltose after 48 h incubation, followed by galactose (881.9 ± 14.25 U/ml). Enzyme production was comparatively less in the medium containing lactose (414.07 ± 16.29 U/ml). In accordance with these several authors have suggested the increasing yield of protease production from many carbon sources, including sucrose (Tsuchiya *et al*., 1997). To evaluate the effect of nitrogen sources on protease production, various nitrogen sources were studied. High yield of enzyme was observed in casein (887.36 ± 14.86 U/ml) and urea (577.01 ± 19.43) substituted medium (Fig 1b). In the earlier reports, casein (1%), gelatine (0.03%), and urea were found to effective supplements for the production of protease for *Bacillus* sp., *B. subtilis* and *B. licheniformis*, respectively (Kamran *et al*., 2015; Pant *et al*., 2014; Suganthi *et al*., 2013). In the present study, among various metal ions tested, enzyme production was found to be high in the medium containing 0.1% magnesium chloride (285.4 ± 9.32 U/ml), followed by zinc chloride (275.4 ± 6.4 U/ml) (Fig 1c). In accordance with the present findings, Feng *et al*. (2001) and Kalaiarasi and Sunitha (2009) have reported that Mg^{2+} and Ca^{2+} influenced more protease production by *B. pumilus* and *Pseudomonas fluorescens*.

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ISSN 2278-7763

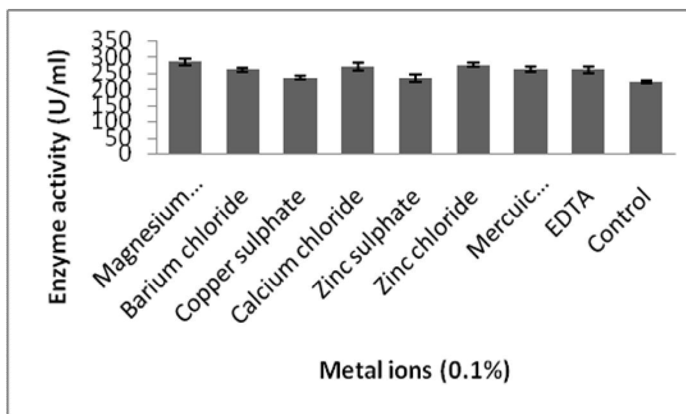

Fig. 1c. Effect of metallic ions on enzyme production by *B. subtilis* AP-CMST W3

The effect of sodium chloride on enzyme production is given in Fig 2a. Maximum (392.9 ± 16.38 U/ml) enzyme production was observed in the culture medium containing 3% NaCl after 48 h incubation, however, the enzyme production was gradually decreased when salt concentration increases above 3%. Esakkiraj *et al*. (2007) have reported an enhanced protease production by *B. cereus* recorded in 3% (w/v) sodium chloride level. The effect of pH and incubation temperature of the culture medium is critical factors and need to be optimized.

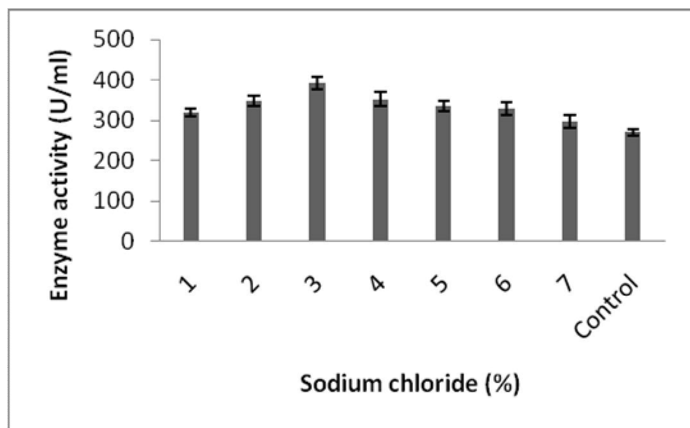

Fig. 2a. Effect of different concentrations of sodium chloride on enzyme production by *B. subtilis* AP-CMST W3.

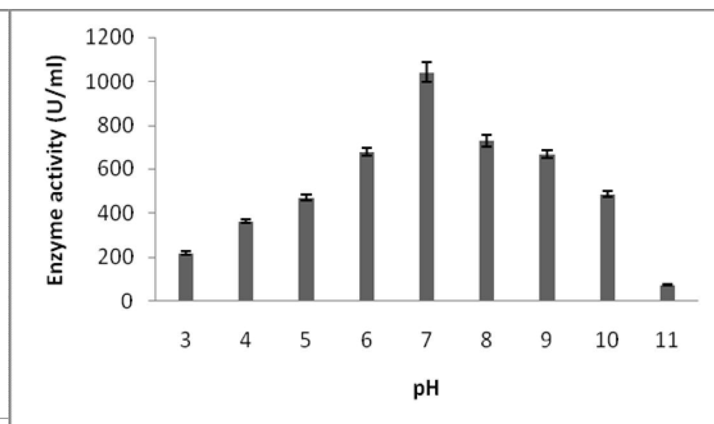

Fig. 2b. Effect of various pH on enzyme production by *B. subtilis* AP-CMST W3.

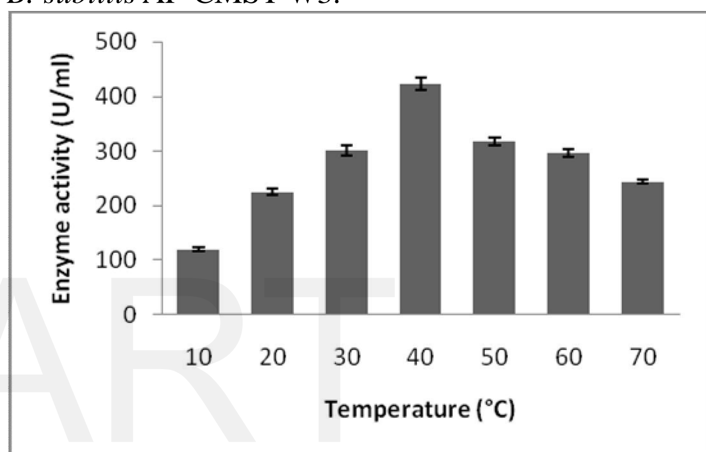

Fig. 2c. Effect of various temperature on enzyme production by *B. subtilis* AP-CMST W3.

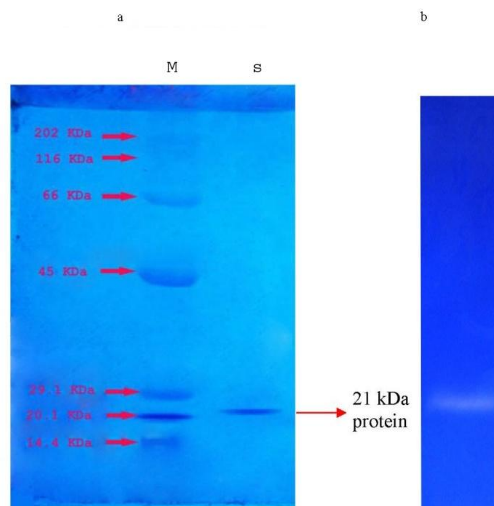

Fig. 3. SDS-PAGE and Zymography analysis of protease from *B. subtilis* AP-CMST W3.

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ISSN 2278-7763

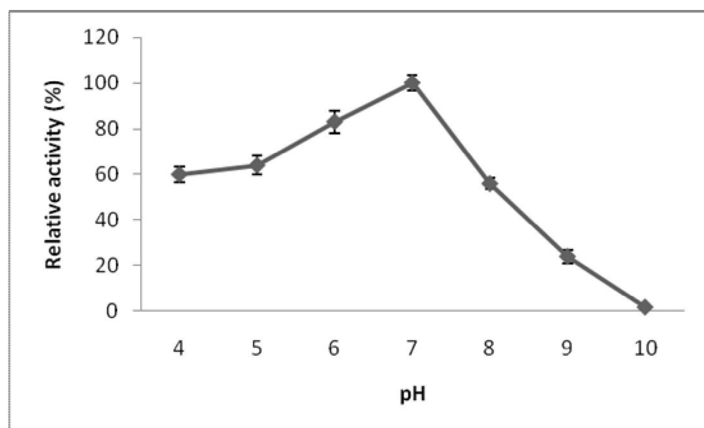


Fig. 4a. Effect of pH on relative activity of protease from *B. subtilis* AP-CMST W3.

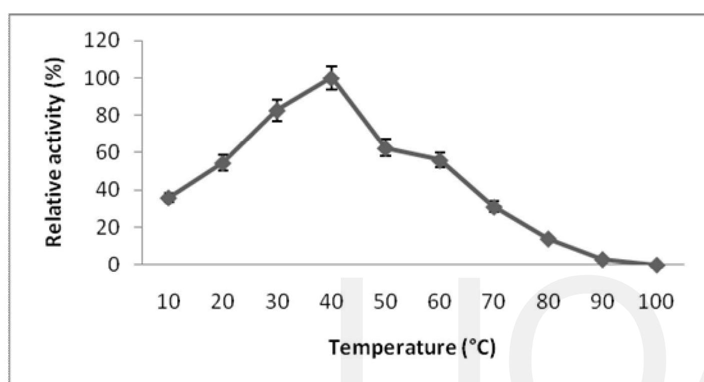
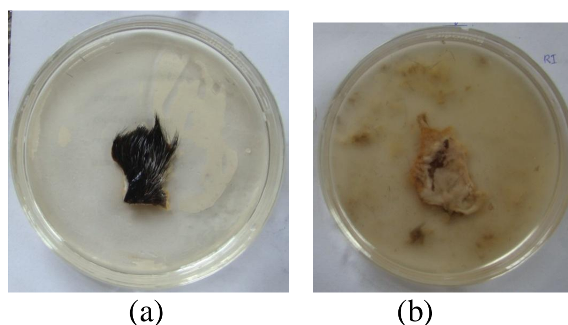


Fig. 4b. Effect of temperature on relative activity of purified protease from *B. subtilis* AP-CMST W3.

In the present study, pH optimization study revealed that the candidate organism *Bacillus subtilis* AP-CMST W3 could produce enzyme over a pH range between 6.0 and 8.0. Maximum enzyme production was observed at pH 7.0 (1042.1 ± 46.35 U/ml) (Fig 2b). Several researchers reported previously that most of the *Bacillus* sp. have optimum pH for the production of protease was in between 7.0 and 11.0 (Shivanand and Jayaraman, 2009). The effect of incubation temperature on the enzyme production was shown in Fig 2c. The optimum temperature for enzyme production by the candidate strain was found to be at 40 °C, when the temperature level increased, it led to decrease the enzyme production. The incubation temperature could regulate and synthesise extracellular protease by micro-organisms. The temperature optimum of the candidate species (40 °C) was found to be high than protease production from *Bacillus* sp. (Gouda *et al*., 2006).



(a) (b)

Fig. 5a. Dehairing of goat skin by protease of *B. subtilis* (a) Control; (b) After 24 h of incubation.

(a) (b)

Fig. 5b. Effect of *B. subtilis* protease on 'X' ray film (a) control, (b) After 24 h incubation (experimental plate).

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The crude enzyme obtained was purified by using ammonium sulphate precipitation, dialysis and sephadex G-75 gel filtration chromatography. The specific activity of crude enzyme was detained as 39.5 ± 3.13 U/mg but after the final purification process, the specific activity increased to 847.8 ± 4.98 U/mg, similarly the purification fold also increased from 1.0 to 21 ± 0.12 purity (Table 2). The molecular weight of the purified enzyme synthesized by *B. subtilis* AP-CMST W3 was estimated as 21 kDa by using the molecular weight marker through SDS-PAGE analysis (Fig 3). This results was in accordance with the findings of Sousa *et al.* (2007), who stated that the molecular masses of most proteases derived from *Bacillus* sp. are less than 50 kDa. In the present study, the optimum pH was found to be 7.0 for maximum enzyme activity (Fig 4a). This result was in accordance with the observations made with proteases from *B. laterosporus* (Usharani and Muthuraj, 2009). The temperature optimum of protease from the candidate organism was lower than protease from *Bacillus anthracis* S-44 (Ammar *et al*., 1991). The protease isolated from AP-CMST W3 was active at 30 – 40 °C (Fig 4b). The protease enzyme was highly stable in the presence of surfactants like SDS, PEG and Triton X-100 (Table 3a). The enzyme was stable against various detergents like

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ISSN 2278-7763

Table 2. Summary of purification of protease from *B. subtilis*

Purification steps	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	1980±132	78400±271	39.5±3.13	1.0	100
Ammonium sulphate precipitation (80%)	468±13.4	30640±209.1	65.4±1.78	1.7±0.09	39±0.02
Sephadex G-75	12.5±0.93	10598±187.42	847.8±4.98	21±0.12	13.5±0.08

Table 3a. Effect of protease on the activity of surfactants.

Surfactants (1%)	Relative activity (%)
SDS	142.00 ± 8.74
Tween 20	101.50±5.76
Tween 40	113.60±7.20
Tween 60	107.30 ±6.98
Tween 80	101.30 ±8.36
Triton X 100	118.80±5.49
PEG	136.30±7.46
Control	100.00±0.00

Ariel (99.3 ± 36%), Sun light (89.4 ± 5.2%) and Tide (85.6 ± 5.84%) (Table 3b). The enzyme stability towards various detergents like SDS is significant because SDS-stable protease are not available except proteases from few bacterial sp. such as *Bacillus* sp. RGR-14 (Oberi *et al*., 2001), *B. clausii*-52 (Joo *et al*., 2003). The properties of protease from candidate organism towards detergents make them more valuable, because many garment industries suggest washing at room temperature (30 ± 2 °C) (Venugopal and Saramma, 2006).

Table 3b. Effect of protease on the activity of detergents.

Detergents (1%)	Relative activity (%)
Ariel	99.30±4.36
Sun light	89.40±6.52
Mr. White	85.10±5.35
Henko	78.30±6.40
Ujala	46.40±2.15
Tide +	85.60±5.84
Surf excel	48.90±2.23
Control	100.00±0.00

In the present study, complete dehairing was achieved after 24 h of incubation goat skin with protease (Fig 5a). In the control skin, no hair loss was observed after 24 h of incubation. Sivasubramanian *et al*. (2008) reported that the protease produced by *B. subtilis* had potent dehairing activity, and it removed hair within 24 h of incubation at room temperature. Aravindan *et al*. (2007) have also reported the dehairing property of protease from *B. cereus* and this was achieved within 24 h of incubation. Huang *et al*. (2003) and Ibrahim *et al*. (2011) demonstrated the dehairing activity of protease from *Bacillus pumilus* with 300 U and 2 U of enzyme / gram skin. Application of crude extracellular lysate of *B. subtilis* AP-CMST W3 resulted in hydrolysis of gelatine layer of X-ray film and silver particles were stripped off from gelatine layer (Fig 5b). Nakiboglu *et al*. (2001) used the enzyme extract obtained from *Bacillus subtilis* ATCC6633 registered optimum activity at neutral pHs and applied in silver recovery process. Recently, Verma *et al*. (2014) extracted proteases from *Thermoactinomyces* sp. RS1 which was reported to use in silver recovery process. Besides its activity towards surfactant, and solvent, the protease from *B. subtilis* AP-

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ISSN 2278-7763

CMST W3 was proven to be suitable for dehairing applications and leather processing industries.

4 CONCLUSION

Proteases produced by *Bacillus* species have significant applications in food industry, leather processing and detergent industry. In the present study, the optimal production, purification, characterization of a new protease produced by the estuarine bacterial isolate, *Bacillus subtilis* AP-CMST W3 was carried out. The enhanced production of protease was achieved by the supplementation of 0.5% maltose, 0.5% casein, 0.1% magnesium chloride, 3% sodium chloride, at pH 7.0 and 40 °C. The purified enzyme has gelatinase activity and removed the gelatine layer of "X" ray film. This enzyme also possesses stability with various commercial detergents. Further, this enzyme can be used in enzymatic silver recovery processes and in detergent industry.

References

[1] M.S. Ammar, S. S. El-Louboudy, and U.M. Abdulraouf, "Protease (s) from *Bacillus anthracis* S-44 and *B. cereus* var. mycoids, S-98 isolated from a temple and slaughter house in Aswan city," *Az. J. Microbiol*., 3: 12-29, 1991.

[2] *R. Aravindan, S. Saravanabhavan, P. Thanikaivelan, J.R. Rao, and B.U.A. Nair,* "Chemo enzymatic pathway leads towards zero discharge tanning," *J. Clean. Product., 2007,* 15: *1217-1227.*

[3] U.C. Banerjee, R.K. Sani, W. Azmi, and R. Soni, "Thermostable Alkaline Protease from *Bacillus brevis* and Its Characterization as a Laundry Detergent Additive," *Process Biochem.,* 35: 213-219, 1999.

[4] B. Bhunia and A. Dey, "Statistical Approach for Optimization of Physiochemical Requirements on Alkaline Protease Production from *Bacillus licheniformis* NCIM 2042," *Enzyme Res.*, 2012: Article ID 905804, 13 pages, 2012. doi:10.1155/2012/905804.

[5] P. Esakkiraj, G, Immanuel, M. Sowmya, P. Iyapparaj and A. Palavesam,"Evaluations of protease producing ability of fish gut Isolate *Bacillus cereus* for Aqua Feed. *Food Bioprocess Technol. Spring. Sci.,* 2:383-390, 2007.

[6] Y.Y.Feng, W.B. Yang, S.L. Ong, J.Y. Hu and W.J. Ng WJ,"Fermentation of starch for enhanced alkaline protease production by constructing an alkalophilic *Bacillus pumilus* Strain. *Appl. Microbiol. Biotechnol.,* 57:153-160, 2001.

[7] S. George, V. Raju M.R.V. Krishnan, T.V. Subramanian and K. Jayaraman, "Production of protease by *Bacillus amyloliquefaciens* in solid-state fermentation ant its application in the unhairing of hides and skins," *Process Biochem.,* 30:457–462, 1995.

[8] M. K. Gouda, "Optimization and purification of alkaline proteases produced by marine *Bacillus* sp. MIG newly isolated from eastern harbor of Alexandria," Pol. J. Microbiol., 55: 119-126 (2006)

[9] R. Gupta, Q. K. Beg and P. Lovenz, "Bacterial alkaline Proteases. Molecular approaches and industrial applications, *Appl. Microbiol. Biotechnol.,* 59: 15-32, 2002.

[10] Q. Huang, Y. Peng, X. Li, H. Wang and Y. Zhang," Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus,"Curr. Microbiol*., 46: 169-173, 2003.

[11] Ibrahim, K. Syed, J. Muniyandi and S.K. Pandian, "Purification and Characterization of Manganese-Dependent Alkaline Serine Protease from *Bacillus pumilus* TMS55," *J. Microbiol. Biotechnol.,* 21(1): 20–27, 2011.

[12] H.S. Joo, G.C. Kumar, G.C. Park, S.R. Paik, and C.S. Chang CS, "Oxidant and SDS stable alkaline protease from *Bacillus clausii* I-52: Production and some properties. *J. Appl. Microbiol*., 267-272, 2003.

[13] F. S. Josephine, V.S. Ramya, D. Neelam, S.B. Ganapa, K.G. Siddalingeshwara, N. Venugopal and T. Vishwanatha, " Isolation, production and characterization of protease from *Bacillus* Sp isolated from soil sample" . *J. Microbiol. Biotechnol. Res.,* 2(1): 163-168, 2012.

[14] A. Kamran, Z. Bibi and M. Kamal,"Screening and enhanced production of protease from a thermophilic *Bacillus* species," *Pak. J. Biochem. Mol. Biol.,* 48(1): 15-17, 2015.

[15] K. Kalaiarasi and P.U. Sunitha. Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil. *Afr. J. Biotechnol.,* 8:7035-7041, 2009.

[16] U. K. Laemmli, "Cleavageofstructuralproteinsduringassemblyofheadof bacteriophageT4," *Nature.* 227, 1970.

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[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, "Protein measurement with the Folin phenol reagent," *J. Biol. Chem.,* 193: 265-275, 1951.

[18] T. Mothe and V.R. Sultanpuram,"Production, purification and characterization of a thermotolerant alkaline serine protease from a novel species *Bacillus caseinilyticus,"3 Biotech*., 6(1): 53, 2016.

[19] N. Nakiboglu, D. Toscali, and I. Yasa, "Silver recovery from waste photographic films by an enzymatic method. *Turk. J. Chem.,* 25: 349-353,2001

[20] R. Oberoi, Q. K. Beg, S. Puri, R. K. and Saxena and R. Gupta R, "Characterization and wash performance analysis of an SDS-resistant alkaline protease from a *Bacillus* sp.," *World. J. Microbiol. Biotechnol.* 17:493-97, 2001.

[21] G. Pant, A. Parakash, J.V.P. Pavani, S. Bera, G.V.N.S. Deviram, A. Kumar, M. Panchpuri and R.G. Prasuna,"Production, optimization and partial purification of protease from *Bacillus subtilis*,". *J. Taibah. Univ. Sci.*, 2014; http://dx.doi.org/10.1016/j.jtusci.2014.04.010

[22] R.S. Prakasham, C.S. Rao and P.N. Sarma,"Green gram husk-an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation. *Bioresour. Technol.,* 97: 1449-1454, 2006.

[23] S.K. Rai and A.K. Mukherjee, "Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04," *Biochem. Eng. J.,* 48(2): 173-180, 2010.

[24] S.K. Rai , A.K. Mukherjee, "Optimization of production of an oxidant and detergent-stable alkaline β-keratinase from *Brevibacillus* sp. strain AS-S10-II: Application of enzyme in laundry detergent formulations and in leather industry," *Biochem. Eng. J.*, 54(1): 47-56, 2011.

[25] P. K. Sarker, S. A. Talukdar, P. Deb, S.M.A. Sayem and K. Mohsina, "Optimization and partial characterization of culture conditions for the production of alkaline protease from *Bacillus licheniformis* P003," *SpringerPlus,* 2: 506, 2013.

[26] N. Sevinc and E. Demirkan, "Production of Protease by *Bacillus* sp. N-40 Isolated from Soil and Its Enzymatic Properties," *J. Biol. Environ. Sci.,* 5(14): 95-103, 2011.

[27] P. Shivanand, and G. Jayaraman, "Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. *Process Biochem.,* 44: 1088-1094, 2009.

[28] Sivasubramanian S, Murali Manohar B, Rajaram A, Puvanakrishna R. Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease. *Chemosphere*. 2008;70:1015–1024.

[29] F. Sousa, S. Jus, A. Erbel, V. Kokol, A. Cavaco-Paulo, and G. M. Gubitz, "A Novel metalloprotease from *Bacillus cereus* for protein fibre processing," *Enzy. Microb. Technol*., 40: 1772-1781, 2007.

[30] C.h. Subba Rao, T. Sathish, P. Ravichandra and R. S. Prakasham, "Characterization of thermo- and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications," *Process Biochem.,* 44: 262–268, 2009.

[31] Sundararajan, C.N. Kannan and S. Chittibabu, "Alkaline protease from *Bacillus cereus* VITSN04: Potential application as a dehairing agent," *J. Biosci. Bioeng.*, 111(2):128-33, 2011.

[32] C. Suganthi, A. Mageswari, S. Karthikeyan, M. Anbalagan, A. Sivakumar and K.M. Gothandam, " Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments," *J. Gen. Eng. Biotechnol.,* 11(1): 47–52, 2013.

[33] H. Takami, T. Akiba and K. Horikaoshi, "Production of extremely thermostable alkaline protease from *Bacillus* Sp. No. AH-101," *Appl. Microbiol. Biotechnol*., 30: 120-124, 1989.

[34] S. Tobe, Y. Nagoh, T. Watanabe, T. Mukaiyama, "Bacteriolytic activity of detergent protease and its enhancement by detergents materials," *J. Oleo Sci.,* 54: 389-395, 2005.

[35] H. Tsujibo, K. Miyamoto, T. Hasegawa and Y. Inamori, "Purification and characterization of two alkaline serine proteases produced by an alkalophilic actinomycete", *J. App. Bacteriol*., 69: 520–529, 1990.

[36] B.Usharani and M. Muthuraj," Production and

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International Journal of Advancements in Research & Technology, Volume 5, Issue 6, June-2016
ISSN 2278-7763

Characterization of Protease Enzyme from *Bacillus laterosporus*," *Global J. Mol. Sci.,* 4(2): 180-186, 2009.
[37] M. Venugopal, A.V. Saramma, "Characterization of alkaline protease from *Vibrio fluvialis* strain VM10 isolated from a mangrove sediment sample and its application as a laundry detergent additive," *Process Biochem.,* 41: 1239–1243, 2006.
[38] H. Varela, M.D. Ferrari, L. Belobradjic, A. Vazquez and M.L. Loperena ML, "Skin unhairing proteases of *Bacillus subtilis*: production and partial characterization," *Biotechnol. Lett.,* 19:755–758, 1997.
[39] A.Verma, M.W. Ansari, M.S. Anwar, R. Agrawal, S. Agrawal," Alkaline protease from *Thermoactinomyces* sp. RS1 mitigates industrial pollution," *Protoplasma.*, 251(3):711-718, 2014.
[40] K.M. Vijay, D. Jesse, F. Aitken, A. Carvalho, C.C. Tyler, M. Simon, S. Shanthi, V.S. Shanthi, K. Rob, E.L. Ruth and T.G. Andrew, "Metabolic Syndrome and Altered Gut Microbiota in Mice Lacking Toll-Like Receptor," *Science*, 5:228-328, 2010.
[41] Q. Yossan, Y. Peng, X. Wang, H. Li and Zhang, Y, "Purification and characterization of an extracellular alkaline serine protease with dehairing Function from *Bacillus pumilus*. *Curr. Microbiol.,* 46: 169- 173, 2006.

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