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Potential of *Bacillus subtilis* to produce acidic protease under mutagenic condition

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ABSTRACT

Protease from Bacillus subtilis has wide applications in industrial sectors.A total of 28 bacteria were isolated from marine mud sample, of which 3 isolates exhibited constructive results for protease production in skim milk, gelatin and casein agar. Among three isolates, MMK 2/8 had superlative enzymatic activity in skim milk broth (170 U/mL) and was subjected for taxonomical identification, which was found to be Bacillus subtilis. The finest conditions were found to be at pH 6.0, temperature 40 °C and 1% NaCl concentration and their maximal enzyme activitywas 354 U/mL, 437 U/mL and 263U/mL respectively, at 36 hrs of incubation. The molecular weight of the protease enzyme was considered to be approximately 50 kDa by SDS-PAGE. The enzyme activity was enriched by exposing it to ultra violet radiation [UV] for 60 secand it was observed to be 610 U/mL. Hence, we charted that the production of acidic protease treated with ultra violet radiation was maximum when compared with the parental strain.

Key words: *Acidic protease, Bacillus subtilis, UV radiation, skim milk agar*

INTRODUCTION

Protease is an enzyme that chiefly involved in cleaving the long peptide chains into short fragments and is produced by various sources such as plants, animals, and microorganisms. Among these, microorganisms were noteworthy,since it can grow in a shorter period, produce a specialized enzyme, cheaper and reliable[1]. In culture medium, large amount of extra cellular proteases is produced by the bacteria which belong to genus *Bacillus*, they are largely present in the soil and mud regions[2, 3]. Marine microorganism producing protease has upraised magnetism by the reason of habituation to extreme conditions[4]. Protease enzyme finds application in various fields like meat tenderization, detergents, silver recovery, laundry, stone washing jeans, pulp and paper manufacture, leather de-hairing and tanning, de- sizing of textiles and de-inking of paper[5, 6]. Hence the yield of protease can be enhanced by exposing the strain to ultraviolet radiation and is universally practiced for inducing strain improvement [7]. Our present study mainly focused on to isolate protease producing strain and tooptimize the cultural condition such as temperature, pH, and fermentation period along with exposure of strains to ultraviolet radiation to investigate the increased protease activity.

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MATERIALS AND METHODS

Sample collection

The mud sample was collected from the coastal region of Manamelkudi, Pudukkottai, Tamil Nadu, India. The sample was collected in sterile containers, then transported to our laboratory to store (-20 deep freezer) before further processing.

Screening and identification

The collected sample was subjected to serial dilution and the individual colonies were checked for protease production using different supplements; skim-milk, casein and gelatin by radial well diffusion agar plate method, and enzymatic assay. The highest protease producers among the isolates were further taken for biochemical and taxonomic identification. For taxonomic identification, the DNA from the strain was isolated using boiling lysis method and amplified using universal primers as forward primer:5'-GAGTTTGATCCTGGCTCAG-3' and reverse primer: 5'-ACGGCTACCTTGTTACGACTT-3'. Species level identification was done at www.ncbi.nlm.nih.gov/BLAST/ and BLAST nucleotide sequence similarity with 98% and above was deemed as sufficient for species identification.

Enzyme assay

Caseinolytic assay was performed to find the activity of protease with some modifications to the existing method. For each set of vials, 5mL of casein solution (0.65%) prepared with potassium phosphate buffer was added and incubated for 5 min at 37^{o}C. Solution was mixed with 1mL of crude enzyme (test or tyrosine), and incubate for 10min at 37^{o}C. 5mL of tri-chloro acetic acid (TCA) was added, incubated for 30 min at 37^{o}C. After incubation, the test solution was filtered using a 0.45 micron syringe filters. Blank was maintained without enzyme solution. Filtrate (2 mL), 5mL of sodium carbonate (Na_2Co_3), 1 mL of Folin's reagent was added, and the mixture was incubated for 30 minutes at 37^{o}C to develop the blue color and read at 660 nm.By taking tyrosine as standard, a calibration curve was plotted. One unit of enzyme activity is defined as the quantity of enzyme that can hydrolyze casein to produce a blue color which is equivalent to 1µmol of tyrosine.

Estimation of protein

The protein estimation was determined by the Lowry method using bovine serum albumin (BSA) as a standard.

Ultra violet (UV) mutagenic studies

For maximum production of enzyme, physical mutagenic agent such as ultra violet irradiation was used[8]. The isolate MMK2/8 was grown in LB broth and in skim-milk at 37^{o}C for 24h. The culture grown in LB broth was subjected to serial dilution from 10^{-1} to 10^{-5} and spread plated on to LB agar and colonies were counted. The culture grew in skim milk broth was taken in sterile petri plate, and was exposed to UV light at different time intervals. The UV treated culture was inoculated in skim-milk broth and enzyme activity was determined after each incubation period.

Impact of pH, temperature and salt concentration towards protease activity

Enzyme activity was analyzed by varying the pH, temperature, and salt concentration in the growth medium. The effect of temperature on protease activity was observed by varying the incubation temperature (35, 40, 45, 50^{o}C), role of pH in the growth medium from pH 5.0 to 9.0. The effect of salt concentration in various percentages from 1% to 5% was also determined by adding NaCl to skim-milk broth media and the enzyme activity was monitored for every 12 hours.

Molecular weight determination

The molecular weight of protease was determined using polyacrylamide gel electrophoresis under denatured conditions in the presence of 12% SDS-PAGE. The molecular weight of protease from *Bacillus subtilis*was verified and checked with available UniProt database. (http://www.uniprot.org/)[9].

RESULTSAND DISCUSSION

Screening and identification of protease producing bacteria

A total of 28 bacteria were isolated from mud samples, and plated on to agar plates (skim-milk, casein, and gelatin), of which 3 isolates were screened, that showed proficient result for protease production with

clear zone. Among the three isolates, MMK2/8 had higher activity of extracellular protease, 170 U/mL after 24h (pH 7.0, 37°C) of incubation in skim-milk broth (Fig.1). For further studies MMK2/8 was selected, and its morphological characterization revealed that the isolate belongs to *Bacillus* sp., (Table 1). By subjecting to taxonomic identification, the isolate was identified as *Bacillus subtilis* (KT760401).

Fig. 1: Total protein contentwas compared with protease activity for three selected isolates (MMK2/6, MMK2/8, MMK2/12) with different nutrient supplement at 37 °C, pH 7.0

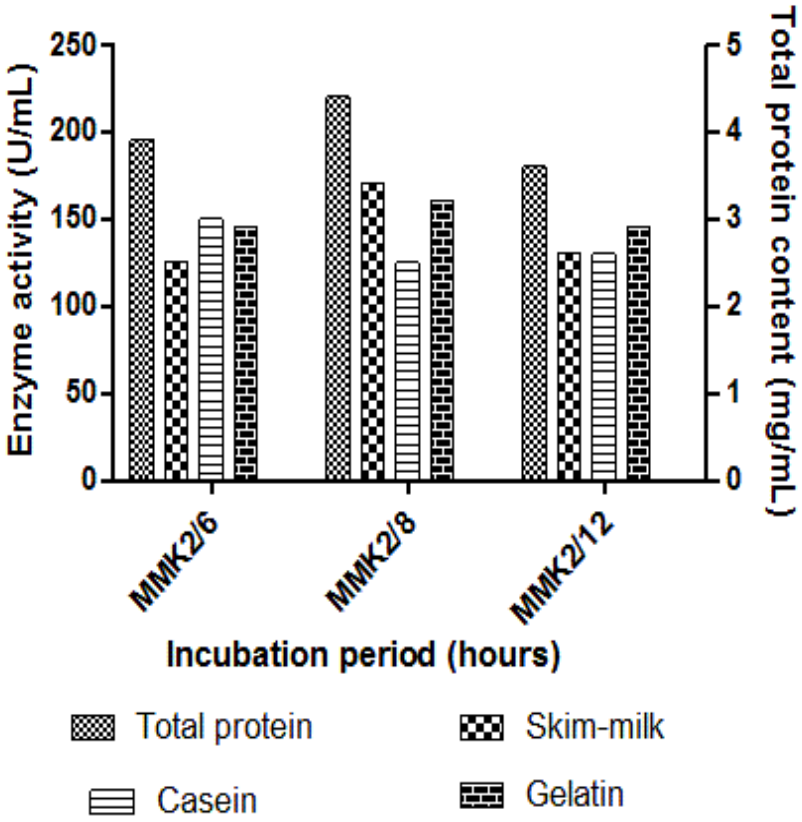


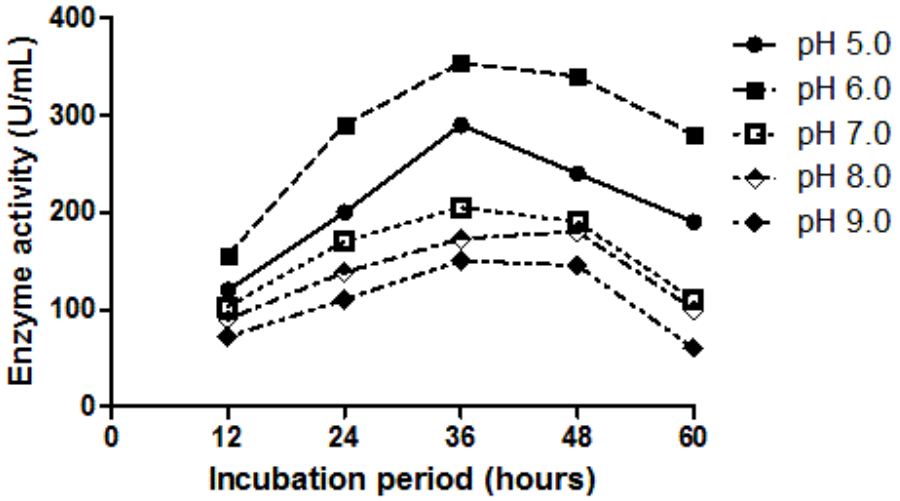
Table 1: Biochemical and morphological characterization of MMK2/8 strain

Characteristic feature	MMK2/8 strain
Colony morphology	Dry, flat, irregular
Gram staining	Gram positive,rod
Hanging drop	Motile
Indole test	Positive
Catalase	Positive
Starch	Positive

Effect of pH on enzyme activity

Optimization with different pH (5.0 – 9.0) revealed that extracellular protease from *B. subtilis* was stable at acidic (pH 6.0) condition. Also pH of the medium had strong influence on enzyme activity (Fig.2). At acidic pH 6.0 maximum enzyme activities were observed, 354 U/mL after 36 h of incubation period. It also revealed that activity was not stable as the pH increase as well as the incubation period. The production of acidic protease plays a vital role in various industrial processes. Inearlier studies, optimum enzyme production was achieved at pH 7.4 and yield is about 10.8 U/mL for every 6 hrs[10].

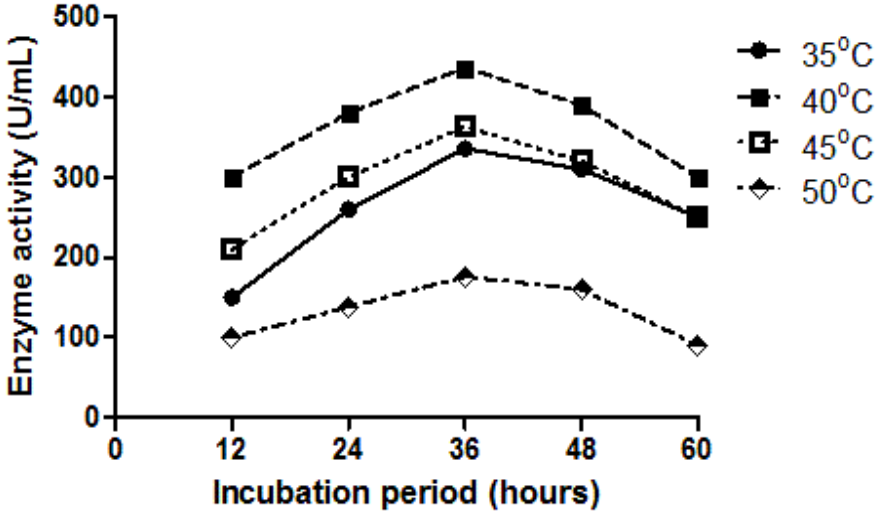
Fig. 2: Effect of different pH on protease activity. For the selected *B. subtilis*, maximum activity was achieved at pH 6.0, after 36h of incubation



Effect of temperature on enzyme activity

Evaluation of protease activity at different temperatures (35-50 °C), showed that extracellular enzyme had maximum activity of 437 U/mL at 40 °C after 36h of incubation. It also showed that increase in temperature gradually decreases the activity of protease (Fig.3). Temperature was found to have profound effect on protease production within *Bacillus* sp. The optimal production of protease was achieved at 40°C to 45°C and the yield of protease varies depending on the medium[11].

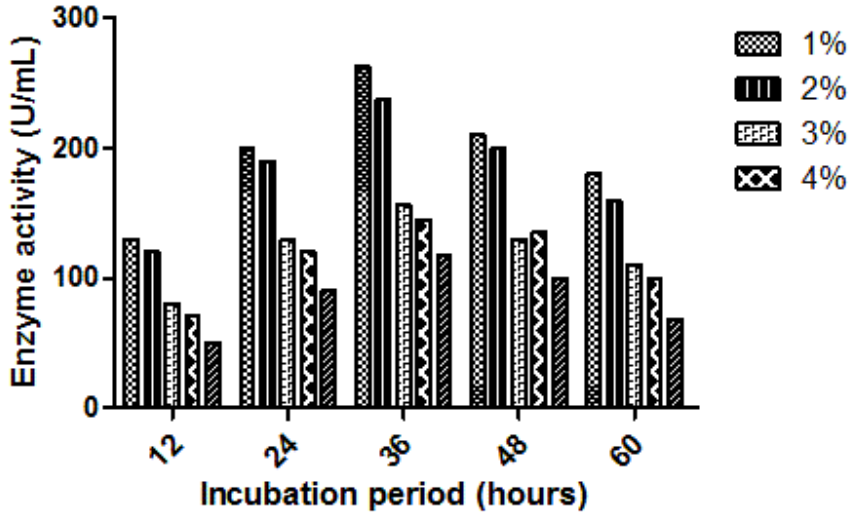
Fig. 3: Impact of incubation period and temperature towards protease activity. *B. subtilis* was found to have maximum activity at 40 °C after 36h of incubation



Effect of salt concentration on enzyme activity

Enzyme activity was found to be decreasing as salt concentration (NaCl) increased from 1% as well as in salt-less medium. Though bacterial growth was observed at higher salt concentrations up to 7%, enzyme activity was inhibited with increasing salt concentration (Fig.4). Accordingly, after 36 hours of incubation 1% NaCl supplemented medium showed an enzyme activity of 263 U/mL. In some studies enzyme secretion was not competent below 5% salt concentration and has optimal activity when the salt concentration is high[12].

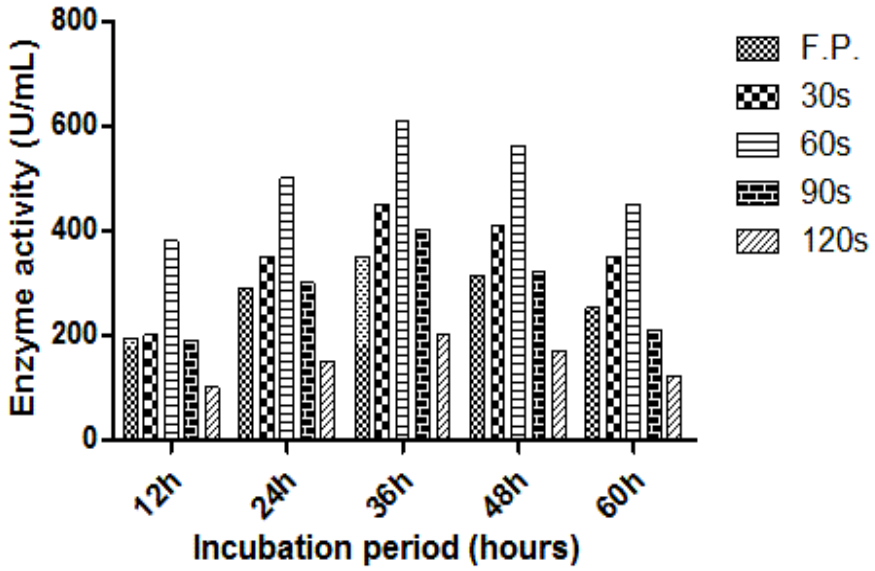
Fig. 4: Enzyme activity at varying salt concentration, 1% of NaCl was found to have strong influence of protease activity



Effect of UV irradiation on enzyme activity

Mutagenic studies with UV exposure at different time intervals revealed that after 60 seconds of UV exposure maximum protease activity was achieved (Fig.5). Mutant strain had maximum activity of 610 U/Ml after 36 h, in contrast parental strain had 351 U/mL at pH 6.0, 40 °C with 1% salt concentration. Similarly, higher activity was observed in the mutant strain than in its parental strain in *B. subtilis*[13,14]. In some cases, UV mutagenesis also inhibited the activity of parental strain that showed lower activity than parental strain in contrast chemical mutagenesis had higher activity[15].

Fig. 5: Effect of physical mutagenesis (UV) towards protease activity

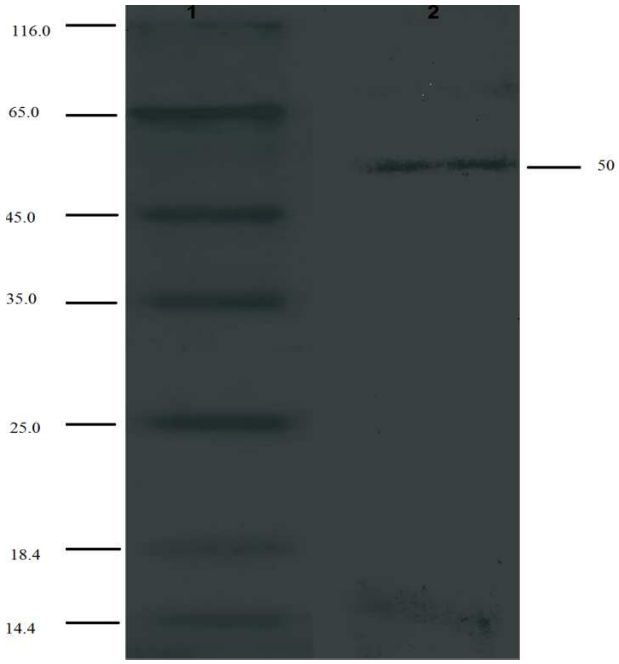


Fermentation period (F.P.) of 36 hours was considered as constant for enzyme assay[pH 6.0, temperature 40 ^{o}C and salt concentration 1%] and protease activity at different intervals (30s, 60s, 90s, 120s) of UV exposure was monitored.

Molecular weight determination

SDS-PAGE analysis showed a molecular mass to be approximately 50 kDa(Fig.6) from *B. subtilis*. Protease from *Bacillus* sp. was found to have molecular weight ranging from 20-60 kDa.Molecular weight for different classes of protease from *B. subtilis* was compared using UniProt tool (Table 2).

Fig. 6: SDS-PAGE results for protease from *Bacillus subtilis*



Lane:1- Protein marker, 2-crude extract. Molecular weights were represented in kDa and found to be ~50.0 kDa.

Table 2: *Bacillus subtilis* protease (Search was performed using network service UniProt)

S.No.	Protein name	UniProt Accession number	Mass of extracellular protein,kDa
1.	Uncharacterized zinc protease YmxG	Q04805	45.995
2.	Uncharacterized zinc protease YmfH	031766	48.949
3.	Uncharacterized serine protease YyxA	P39668	42.789
4.	Carboxy terminal processing protease	034666	51.149
5.	Neutral protease B	P39899	59.336
6.	Minor extracellular protease vpr	P29141	85.60
7.	Uncharacterized protease YdeA	P96658	22.454
8.	Putative cysteine protease YwpE	P94587	11.468
9.	Protease PrsW	P50738	24.719
10.	Rhomboid protease GluP	P54493	56.462

CONCLUSION

This study impliesthat the bacteria isolated from marine mud sample have the ability to produce acidic protease and can widely be used in industries for various applications. For improving the productivity of protease, the isolated *Bacillus subtilis* were subjected to physical mutagenic conditions. Its optimal production by the parental strain was deficient when compared to mutate strain. Further this acidic protease can be purified and utilized for industrial purposes.

REFERENCES

1. Burhan, A., Nisa, U., Gokhan, C., Ashabil, A. and Osmair, G., Enzymatic Properties of a novel thermostable thermophilic alkaline and chelator resistant amylase from an alkaphilic Bacillus spisolate ANT-6, *Process Biochemistry*, **38:** 1397–1403(2003).
2. Dubal,S.A., Tilkari,Y.P.,Momin,S.A., Borkar,I.V.,Biotechnological routes in flavour industries,*Biotechnology advances*, **6:** 30–45(2008).

3. Sekhon, B.S., Food nanotechnology— An overview, *Nanotechnology Science and Applications*, **3:** 1–15 (2010).
4. Jayraman, G., Shivanand, P., Isolation and characterization of a metal ion – dependent alkaline protease from a halotolerant Bacillus aquimaris VITP4.,*Indian Journal of Biochemistry and Biophysics*, **48:** 95-100 (2011).
5. Enzyme Technical Association. Enzymes; A primer on use and Benefits today and tomorrow. Washington, DC 2000 **36:** 1-32 (2001)
6. Gupta, R., Beg, Q.K., Lorenz, P., Bacterial alkaline proteases: molecular approaches and industrial application. *Applied Microbiology and Biotechnology*, **52:** 15–32 (2002).
7. Basavaraju, S., Kathera, C., Jast, P.K., Induction of Alkaline Protease Production by Bacillus Mutants Through U.V. Irradiation, *International Journal of Pharmaceutical Sciences Review and Research*, **12:** 78-83(2014).
8. Saha, N., & Bhattacharya, B.C., Strain improvement mutagenesis and random screening procedure for Rhizopusoryzae IIT KG-1., *Proceeding of International symposium on industrial biotechnology*, (1990)
9. Gustin, J.K., Kessler, E., Ohman, D.E.,A substitution at His-120 in the LasA protease of Pseudomonas aeruginosa blocks enzymatic activity without affecting propeptide processing or extracellular secretion. *Journal of Bacteriology*, **178(22):** 6608-17 (1996).
10. Kumar,R., Vats,R., Protease production by Bacillussubtilisimmobilized on different matrices, *New York Science Journal*, **3:** 20–24(2010)
11. Maghsoodi,V., Kazemi,A., Nahid,P., Yaghmaei,S., Sabzevari, M.A., Alkaline protease production by immobilized cells using B. licheniformis, *Science Iran*, **20:** 607–610 (2013).
12. Rajesh Patel , MitalDodia , Satya P. Singh., Extracellular alkaline protease from a newly isolated haloalkaliphilic Bacillus sp., Production and optimization, *Process Biochemistry*,**40:** 3569–3575(2005).
13. Puri, S., Beg, Q. K. and Gupta R., Optimization of alkaline protease production from *Bacillus* species by response surface methodology, *Current Microbiology*, **44:** 286-290 (2002).
14. Wang, H. Y., Liu, D. M., Liu Y., Cheng, C. F., Ma, Q. Y., Huang, Q. and Zhang, Y. Z., Screening and mutagenesis of a novel Bacillus pumilus strain producing alkaline protease for dehairing., *Letters in Applied Microbiology*, **44:** 1-6(2007).
15. Solaiman, E.A.M., Wafaa, K. HegazyMaysa, E and Moharam., Induction of over producing alkaline protease Bacillus mutants through U.V irradiation,*Arabian Journal of Biotechnology*, **8:** 49-60 (2004).