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Exploration of Alkaline Protease from Marine Bacillus cereus as a Biocatalyst for Enhanced Drug Delivery Application

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ABSTRACT: Proteases performance an important role in biomedical in pharmaceutical applications, Mainly in the design of progressive drug delivery systems and controlled release formulations. In the present study, marine-derived microorganisms were explored as a potential source of alkaline protease with relevance to drug delivery technologies. Environmental samples collected from waste-contaminated marine regions of Kodungaiyur were partitioned for proteolytic activity using skim milk agar. Among isolated, A1, B2, and C2 exhibited significant hydrolysis zones, with isolate A1 showing the highest activity (21mm). Molecular identification through 16S rRNA sequencing, along with biochemical and morphological characterization, confirmed isolate A1 as *Bacillus cereus*. Optimization of fermentation conditions consuming the One Mutable at a Time (OVAT) approach revealed maximum protease production at pH 7.0, 40 °C, and 76 hours of incubation, yielding an activity of 710.03U/mL. Partial purification via ammonium sulfate precipitation and dialysis enhanced the enzyme's specific activity. The protease demonstrated notable stability under alkaline conditions, indicating its suitability for pharmaceutical applications. Importantly, the characterized alkaline protease exhibits promising potential in drug delivery systems, particularly in enzyme-assisted drug release, peptide-based therapeutics, and targeted delivery strategies. Its stability and activity profile suggest applicability in the development of biodegradable and controlled drug delivery platforms. Further studies focusing on nano formulation, immobilization, and in vitro drug release kinetics are recommended to fully explore its utility in advanced drug delivery technologies.

1. INTRODUCTION

Lorem The capacity of proteases to hydrolyze peptide bonds with great specificity and efficiency makes them one of the most researched classes of biocatalysts and accounts for over 60% of the worldwide enzyme market. Beyond their traditional industrial applications, proteases have gained increasing attention in the pharmaceutical field, particularly for their role in drug

delivery systems, where they facilitate targeted drug release, peptide modification, and bioavailability enhancement (Afrin et al.,2024). Among them, alkaline proteases were distinct attention owing to their constancy and activity under alkaline conditions, which are advantageous for designing robust and controlled drug delivery platforms. Marine microbes have been a promising source of new enzymes with distinctive structural and functional characteristics in recent years. The extreme environmental conditions of marine ecosystems such as high salinity, variable pH, and limited nutrients drive the evolution of enzymes with enhanced stability, catalytic efficiency, and adaptability (Anandharaj et al., 2016). These attributes make marine-derived proteases particularly suitable for biomedical applications, including enzyme-responsive drug delivery systems and nanoformulation strategies. Bacillus species widely known for their high production of proteases, capacity for extracellular secretion, and simplicity of growing and genetic modification. Among them, *Bacillus cereus* has shown significant potential source of alkaline protease with desirable biochemical characteristics (Anne et al., 2016). In this context, current study focused on the isolation, optimization, and characterization of alkaline protease from marine-derived *Bacillus cereus* obtained from waste-contaminated coastal environments (Baweja et al., 2016). Fermentation parameters were optimized to enhance enzyme yield, followed by purification and biochemical characterization to evaluate its functional properties. Importantly, this study explores the potential application of the extracted alkaline protease as a biocatalyst in advanced drug delivery systems (Elias et al., 2014). Its stability and catalytic efficiency highlight its suitability for enzyme-assisted drug release, targeted delivery, and the development of controlled release formulations. The findings provide a foundation for further investigation into nano-enabled drug delivery platforms and pharmaceutical formulations incorporating marine-derived proteases.

2. MATERIALS AND METHODS

2.1 Equipment's Used

Avigen Bio Pvt Ltd's laminar air flow chamber, autoclave, centrifuge, double beam spectrophotometer, pH meter, shaker, weighing balance, and other glassware were among the tools used.

2.2 Chemicals Used

Tri-Chloro Acetic Acid (TCA), Folin-Phenol Reagent, Casein, Tris Buffer (PH-8), Sodium Phosphate Buffer, Bromo Phenol Blue, Sodium Hydroxide, Hydrochloric Acid, Bradford's Reagent, and Ammonium Sulphate acquired from Hi Media Laboratories Pvd Ltd in Mumbai.

2.3 Cleaning of Glassware's

Glassware was extensively cleaned with a detergent solution after being submerged in a chromic acid solution (10% potassium dichromate in 25% sulfuric acid) for few hours (6). Glassware once again cleaned using tap water previously dried in a hot air oven.

2.4 Collection of soil sample

A sample of milk industry waste oil was gathered. It is regarded as the source of protease and is used with a sterile spatula. Production measures were implemented to reduce contamination.

2.5 Isolation Of bacteria from soil sample

Using successive dilution agar plate procedures (dilutingupto10-9 dilution) on LB agar plates, bacteria from the oil sample were recovered (Homaei et al., 2022). In order to isolate individual colonies, the mixed cultures were further separated using the spread plate technique and zigzag streaking on nutrient agar plates based on colony morphology.

2.6 Serial Dilution

A serial dilution was series of successive dilutions castoff to lower impenetrable cell culture to an attentiveness that is more practical. The concentration of bacteria lowered by a certain volume in serial dilution soil (Ida et al., 2017). The Ten tubes containing nine milliliters of sterile distilled water are made as dilution blanks. After transferring 1g of soil sample containing the initial bacterial culture to tube 1 and gently swirling tube 1, 1ml from tube 1 is transferred to tube 2. After 10-9 dilution, same process is repeated for the remaining tubes.

2.7 Spread Plate Technique

Microbes on a media plate are called spread plates. The spread plate method can be used to separate, count, and evenly disperse microorganisms in a mixed culture (Imdakim et al., 2015). Test tubes containing 0.1 ml of diluted microorganisms (10-3 to 10-7) are moved to petri dishes with lb agar medium. Alcohol was used to dip the l-rod, or l-shaped glass spreader (Jensen et al., 2010). The samples were then equally distributed throughout the agar surface using the glass spreader, and the plates were incubated at 37°C for a whole day while the petri dish underneath was carefully rotated.

2.8 Streak plate technique

One method for separating pure cultures of organisms from mixed populations was streaking. In order to thin out the bacteria, the inoculation is streaked across the agar surface (Kabense et al., 2019). Discrete colony formation results from certain individual bacterial cells being well-separated from one another. Units (CFU's) single colony obtained by spread plate method was taken in inoculum loop and spread across one quadrant of a sterile petri dish containing LB agar medium (Kumar et al., 2008). Similarly, other colonies were spread on respective petri dish and incubated at 37^0C for 24hrs.

2.9 Screening of protease producing bacteria

The isolated pure strains were screened for the production of extracellular protease production using screening medium such as skim milk agar (Nascimento et al., 2004). The pure cultures were streaked over the sterile milk agar plates and the plates were incubated at 37^OC for 24 hours. The purpose of the observation was to see the area surrounding the colony that was used for substrate (Oliveira et al., 2017). For additional research, only positive and superior zone-formed strains were used.

2.10 Sub-culturing of positive culture

The better and more positive zone-forming bacterial species were then subcultured in LB broth using quadrant streaking on nutrient agar plates and slant preparation (Omidinia et al., 2012). For additional experimental research, the pure culture was collected once a week and kept at 4°C.

2.11 Protease enzyme assay (qualitative analysis)-plate assay

The agar diffusion method used to measure protease activity. The composition of the skim milk agar (SMA) medium was followed in its preparation. Act Oclave was used to sterilize the medium components after they were put to an Erlenm Eyer flask at 121°C (Qureshi et al., 2016). The sterile petri plate was then filled with the SMA medium. A cork borer was used to carve test and control wells onto the media after it had solidified. After adding the centrifuged culture (supernatant) to the test well, it was incubated at 37°C for a full day (Rao et al., 2007). The well's surrounding hydrolytic zone was noted.

2.12 Optimization studies-effect of various parameters on protease production

2.12.1Time

Aseptically, 5% inoculum was introduced to around 500 milliliters of sterile production medium. The infected medium was shaken at a speed of around 150 rpm while being incubated at 37°C (Rehman et al., 2017). About 20 milliliters of culture were aseptically drowned every 12 hours for a maximum of 60 hours after incubation. Measurements were made of the culture filtrate's total protein content and gelatin activity.

2.12.2 Temperature

A 5% inoculum was added to 100 milliliters of sterile production media in a different conical flask. Each flask was incubated at 25, 30, 35, 40, and 45 degrees Celsius for 48 hours. Protein estimate and enzyme activity were computed.

2.12.3 pH

100 milliliters of sterile production medium were made in different conical flasks using 0.1N NaOH and 0.1N HCl. Each flask was then adjusted to a different pH, such as 5, 6, 7, 8, and 9. After sterilization, flasks were inoculated with 5% inoculum (Umayaparvathi et al., 2013). The flasks were incubated at 37ºC for 48 hours. Protein estimate and enzyme activity were computed.

3. RESULTS AND DISCUSSION

3.1 Sample Collection and Microbial Plating

Environmental samples systematically collected from four distinct locations within Kodungaiyur waste treatment zone to explore novel microbial sources of biocatalysts with potential pharmaceutical relevance (Vijayaraghavan et al., 2014). The samples, categorized as A (thick sludge), B (turbid wastewater), C (decomposed organic waste), and D (soil-contaminated effluent), exhibited diverse physicochemical characteristics, which are likely to influence microbial diversity and enzymatic potential. Such heterogeneous environments are recognized as rich reservoirs of microorganisms capable of producing enzymes with unique structural and functional properties suitable for biomedical applications (Beg et al., 2003). Microbial-derived proteases have gained considerable importance in drug delivery technology due to their ability to facilitate enzyme-responsive drug release, peptide modification, and targeted therapeutic action. In this case, skim milk agar, a common qualitative test for identifying proteolytic activity, was used to screen the obtained samples for microorganisms that produce proteases. Protease production was indicated by the creation of distinct zones surrounding active colonies as a result of the hydrolysis of casein in skim milk (Bundela et al., 2013). A total of 16 distinct colonies exhibiting prominent zones of hydrolysis were selected for further evaluation, suggesting significant proteolytic potential. This preliminary screening approach is widely employed for identifying enzyme-producing strains and serves as a crucial step in isolating candidates suitable for pharmaceutical and drug delivery applications (Sevinc et al., 2011). The identified isolates may further be explored for their enzymatic efficiency, stability, and compatibility in developing advanced drug delivery systems, including enzyme-triggered release platforms and nanoformulations.

3.2 Isolation of Protease-Producing Bacteria

Environmental samples serially diluted up to 10^{-9} and plated to isolate distinct microbial populations. Colony morphology, including size, shape, texture, and pigmentation, was carefully examined at each dilution level, and growth patterns were systematically recorded. Well-isolated colonies obtained at the 10^{-6} dilution selected for further analysis grounded on their distinct morphological characteristics (Figure 1) (Prakasham et al., 2006). Particularly in the context of biomedical and pharmaceutical applications. Microbes adapted to extreme or variable environmental conditions are known to produce enzymes with enhanced stability across a wide range of temperatures, pH levels, and salinity. Such properties are highly desirable for designing enzyme-assisted drug delivery systems, where stability and controlled activity are critical (Olajuyigbe et al., 2005). Proteases, a major class of hydrolases, account for a substantial proportion of commercially relevant enzymes and have emerged as key biocatalysts in drug delivery technology. Their ability to hydrolyze peptide bonds enables applications in enzyme-responsive drug release, protein/peptide drug modification, and targeted therapeutic delivery (Kumar et al., 1999). The isolated strains in this study were therefore considered potential candidates for further screening and characterization to evaluate their suitability in advanced drug delivery systems and pharmaceutical formulations.

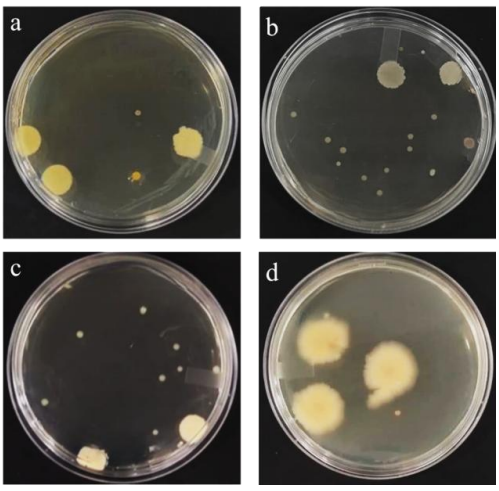


Figure.1 Bacterial isolates from sample sites

3.3 Pure Colony Isolation

A total of eleven unique bacterial colonies were obtained from four different environmental sample locations labeled A, B, C,

and D. As listed in Table.2, of these, locations A, B, and C each produced three different isolates, and site D produced two colonies with diverse morphologies (Jisha et al., 2013). Table displayed the results of an examination of the isolates' colony morphology based on observable traits such as size, color, texture, elevation, and margin. The colonies from Site A were primarily yellow to orange in colour, with complete edges, elevated or convex elevations, and smooth or glossy surfaces (Gupta et al., 2002). Small smooth white colonies, a huge dry creamy-white colony with an undulate edge, and a pinkish-white colony with smooth convex characteristics were among the most variable colonies seen at Site B. The isolates from Site C were mostly white colonies with regular and irregular edges, smooth or dry textures, and sizes ranging from very small to large (Aretz et al., 1989). Large off-white fluffy colonies with filamentous borders and a little pale-white colony with a smooth, flat surface were the two distinct-looking colonies found at Site D. Among this only 8 colonies were subjected to the next stage of study, as few colonies resembled each other.

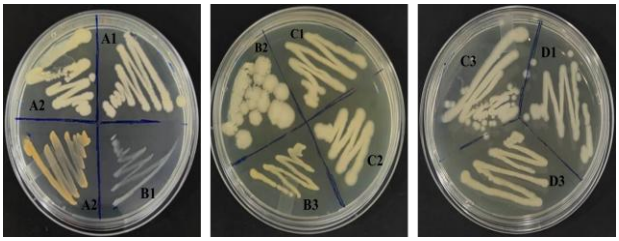


Figure.2 Pure Colony Isolation

Table.1 No of distinct colonies from each site

S. No.	Site	Number of Isolates
1	A	3
2	B	3
3	C	3
4	D	2
Total Number of Isolates		11

Table. 2 Distinct colony morphology of isolates

Isolate Code	Size	Color	Texture	Elevation	Margin
A1	Large	Yellow	Smooth	Raised	Entire
A3	Small	Orange	Shiny/Smooth	Convex	Entire
B1	Smalls	White	Smooth	Convex	Entire
B2	Large	Creamy-white	Dry	Raised	Undulate
B3	Medium	White	Smooth	Convex	Entire
C1	Small–Medium	White	Smooth	Convex	Entire
C2	Large	White	Dry/Wrinkled	Raised	Irregular
D1	Large	Off-white	Fluffy	Raised	Filamentous

3.4 Primary Identification of Isolates by Gram Staining

The bacterial isolates were separated according to the structure of their cell walls using Gram staining. Among eight isolates,

the majority Gram-positive rods. Isolates A1, B2, B3, C1, C2, and D1 retained crystal violet stain and appeared purple under the microscope, confirming them as Gram-positive bacilli (Bascaran et al., 1990). These isolates showed well-defined rod-shaped morphology. Isolates A3 and B1, on the other hand, appeared pink, indicating a Gram-negative reaction (Boyer et al., 1968). B1 displayed coccoid or short rod morphology typical of Gram-negative bacteria. A3 showed pink-stained rods that were loosely arranged. C1 initially appeared ambiguous but was confirmed as Gram-positive based on stain intensity. The predominance of Gram-positive rods suggests a high likelihood of extracellular enzyme-producing bacteria (Cherdyntseva et al., 1982). Because they can secrete proteins directly into the culture medium, which promotes correct folding in contrast to the intracellular environment, gram-positive bacteria are promising hosts for heterologous protein synthesis. Because of signal peptidase cleavage, secreted proteins also keep their original N-terminal sequences (Jeong et al., 2000). Several Gram-positive species have been explored based on their industrial relevance, efficient secretion systems, non-pathogenic nature, and genetic tractability.

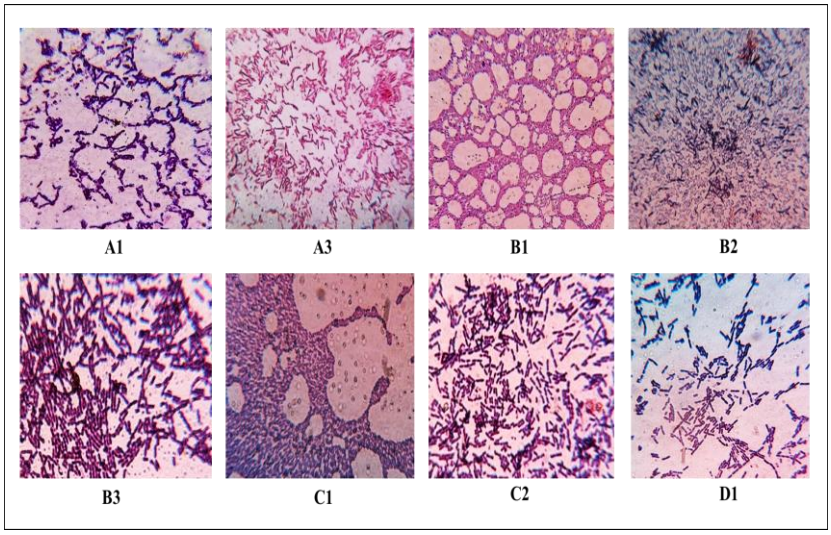


Figure. 3 Isolated of Gram staining

Table.3 Primary Identification of Isolates by Gram Staining

Sl.No	Isolate Code	Gram Reaction	Cell Morphology
1	A1	Gram-positive	Rods (bacilli)
2	A3	Gram-negative	Rods (bacilli)
3	B1	Gram-negative	Coccoid/short rods
4	B2	Gram-positive	Rods
5	B3	Gram-positive	Rods
6	B1	Gram-negative	Coccoid/short rods
7	C2	Gram-positive	Rods
8	D1	Gram-positive	Rods

3.5 Screening of Protease Producing Microorganisms

Skim milk agar was used to assess the eight bacterial isolates' proteolytic activity. The generation of extracellular protease was confirmed by the clear zones surrounding colonies, which showed casein hydrolysis. With a noticeable hydrolysis zone of 21 mm, isolate A1 had the highest activity among the others (AbdelGalil et al., 1992). C2 and B2 had zones of 14 mm and 13 mm,

respectively. Under the measured conditions, the remaining isolates did not produce detectable clearance zones, indicating little or no protease secretion. A1's potential as a high-yielding protease producer is highlighted by the considerable hydrolytic activity that was detected in it (Bhaskar et al., 2007). Clear zone formation indicated protein hydrolysis by proteases from the isolates, with peptone and skim milk serving as carbon sources. Among the strains, BN1 showed the highest proteolytic index (0.64 mm), followed by BN5 (0.54 mm), BN2 (0.51 mm), BN3 (0.24 mm), and BN4 (0.13 mm). The enzymatic index reflects microbial growth rate, enzyme production capacity, and efficiency (Brown et al., 1991). The presence of a clear zone around bacterial colonies signifies their ability to degrade skim milk protein in the medium for growth. Protease enzymes cause this protein depletion by breaking it down into simpler molecules like peptides and amino acids (Buchanan et al., 1974). The three protease positive isolates were shown in Figure. 4 and the diameter of the zone of proteolysis is tabulated in Table .4.

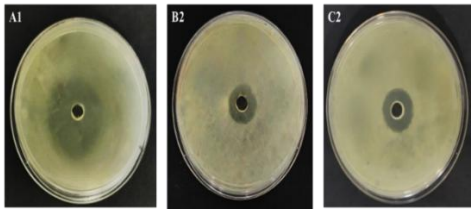


Figure. 4 Screening of Protease Activity

Table.4 zone of proteolysis

Sl. No.	Isolate	Zone of Proteolysis (mm)
1	A1	21
2	B2	13
3	C2	14

3.6 Biochemical Analysis

After that, a number of biochemical analyses were performed on the chosen bacterial sample. The tests for Indole Production, Methyl Red, Voges-Proskauer, Citrate Utilization, and Catalase were performed on the bacterial culture (Chekireb et al., 2009). The results for each test was tabulated and based on their morphological test and biochemical test the bacteria were identified as Bacillus subtilis.

Table. 5 Biochemical Test for Identification of Bacillus subtili

S.No.	Test	Results
1	Indole Production Test	-
2	Methyl Red Test	-
3	Voges-Proskauer Test	+
4	Citrate Utilization Test	+
5	Catalase Test	+

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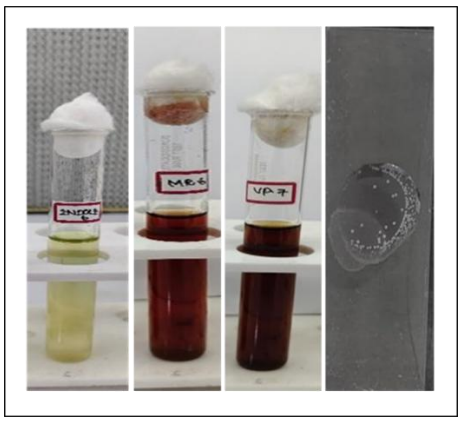


Figure. 5 Biochemical Test (a) Indole Production (b)MR test (c) VP test (d) Catalase Test

3.7 Optimization of Production Process Parameters

An optimization study was conducted using the One Variable at a Time (OVAT) approach, where the effects of pH (5, 6, 7, 8, 9), incubation time (12, 24, 48, 76, 92 hours), and temperature (25°C, 30°C, 35°C, 40°C, 45°C) on the response variable were individually evaluated (Deng et al., 2010). During the pH and temperature trials, the incubation time was fixed at 48 hours, while during the time trials, pH was held at 7.0 and temperature at 35°C.

Table 6 Parameter Optimization

Run	Variable Tested	pH	Time (hr)	Temp (°C)
1	pH	5	48	35
2		6	48	35
3		7	48	35
4		8	48	35
5		9	48	35
6	Incubation time	7	12	35
7		7	24	35
8		7	48	35
9		7	76	35
10		7	92	35
11	Temperature	7	48	25
12		7	48	30
13		7	48	35
14		7	48	40
15		7	48	45

3.8 Enzymatic Assay

The One Variable at Time (OVAT) approach was used to assess the impact of pH, temperature, and incubation duration on protease activity. Tyrosine used as a standard to measure protease activity, as seen in Figure.6. The influence of pH on enzyme

activity revealed that protease production was highest at pH 7.0, yielding 523.95 U/mL, followed by pH 8.0 (324.54 U/mL) and pH 6.0 (296.01 U/mL), while the lowest activity was recorded at pH 5.0 (171.02 U/mL) (Table 4.5, Figure 4.7a). The initial pH of the production medium plays a crucial role in protease synthesis. Microbial growth and enzyme production are highly influenced by the surrounding extracellular pH, as microorganisms rely on it for optimal metabolic activity (Rao and Narasu 2007). Temperature had a significant effect on enzyme activity, with optimal production observed at 40°C (591.18 U/mL). Activity decreased at both lower and higher temperatures, with values of 433.90 U/mL at 45°C and 185.11 U/mL at 25°C (Figure 4.7b, Table 4.6). Temperature is a key factor that affects cellular metabolism and the behavior of biomolecules like proteins. It influences reaction rates, frequency of molecular collisions, the stability of molecular interactions, and other physicochemical properties essential for protein function (Elias et al. 2014). Similarly, incubation time also influenced protease activity, with a peak observed at 76 hours, suggesting that prolonged incubation enhances enzyme secretion up to a certain point, beyond which activity may plateau or decline (Figure 4.7c, Table 4.7). These results indicate that the optimal conditions for maximum protease activity were pH 7.0, 40°C, and an incubation time of 76 hours. Incubation time plays a vital role in defining the phase of maximum enzyme secretion. Any variation from the optimal duration can lead to a decline in protease yield and disrupt the precise control necessary for efficient fermentation-based production(Afrin et al. 2024) utilized Aspergillus fischeri and Penicillium citrinum in submerged fermentation (SmF) using a medium containing 0.5% crushed feathers as a nitrogen source. They reported the production of two collagenolytic serine peptidases. Enzyme assays revealed that A. fischeri produced 460 U/mL at 72 hours, while P. citrinum reached 760 U/mL at 168 hours (Ida et al. 2017). Numerous studies have documented protease production by bacterial strains. For instance, Anandharaj et al. (2016) identified alkaline metalloproteases from Bacillus alkalitelluris TWI3, while Rehman et al. (2017) reported metalloprotease production by Bacillus subtilis KT004404. Similarly, de Oliveira, Rieger, and Daroit (2017) used Bacillus sp. CL33A, and Baweja et al. (2016) reported alkaline protease production by B. pumilus MP 27 under submerged fermentation (SmF). Imdakim et al. (2015) described acidic protease production from Pediococcus acidilactici SH in SmF. Additionally, Jensen et al. (2010) discovered a novel bacterial glutamic peptidase and successfully expressed it heterologously using a B. subtilis expression system. Several Bacillus species have also shown significant protease activity under solid-state fermentation (SSF), including Bacillus sp. BBXS2 with 12,200 U/g activity Qureshi et al. (2016) and B. cereus with 4,813 U/g activity (Vijayaraghavan, Lazarus, and Vincent 2014). Enzymes intended for use in detergents must remain active and stable under alkaline pH, varying ionic strengths of detergent solutions, and across a wide temperature range (25–60°C) (40). Additionally, several other factors are critical in selecting an effective detergent protease, including compatibility with detergent ingredients like surfactants, fragrances, and bleaching agents, as well as efficiency in stain removal and long shelf life (Kumar et al. 2008).

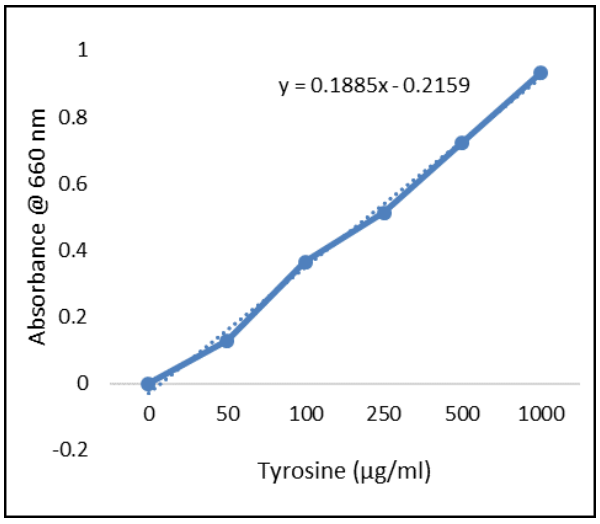


Figure 6 Tyrosine standard curve for determination of protease activity

Table 7 Effect of pH on protease activity

Sl.No	Effect of pH	Protease Activity (U/mL)
1	5	171.02
2	6	296.01
3	7	523.95
4	8	324.54
5	9	247.33

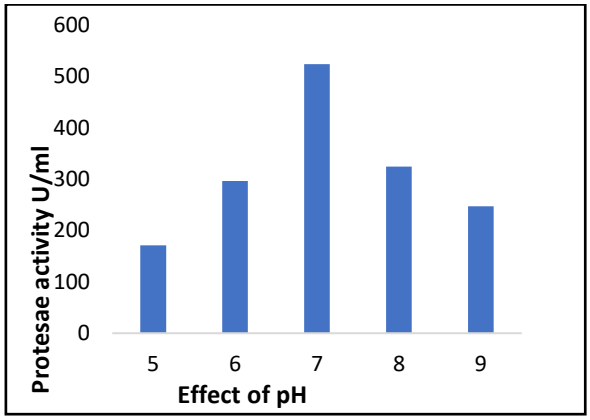


Figure 7 Effect of pH on protease activity

Table 8 Effect of temperature on protease activity

S.No	Effect of temperature	Protease Activity (U/mL)
1	25	185.11
2	30	269.51
3	35	374.20
4	40	591.18
5	45	433.90

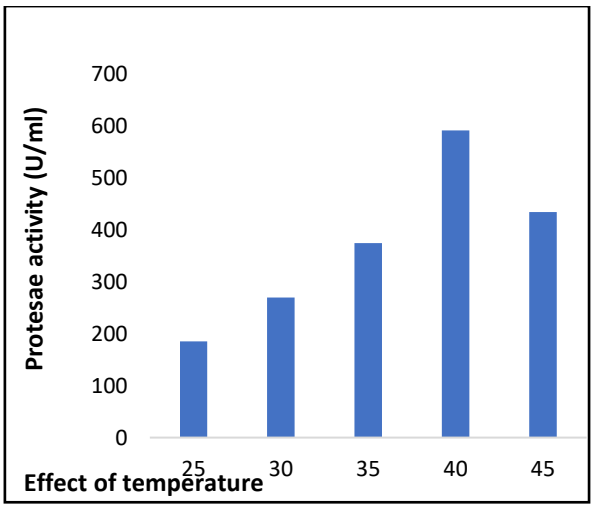


Figure 8 Effect of temperature on proteas activity

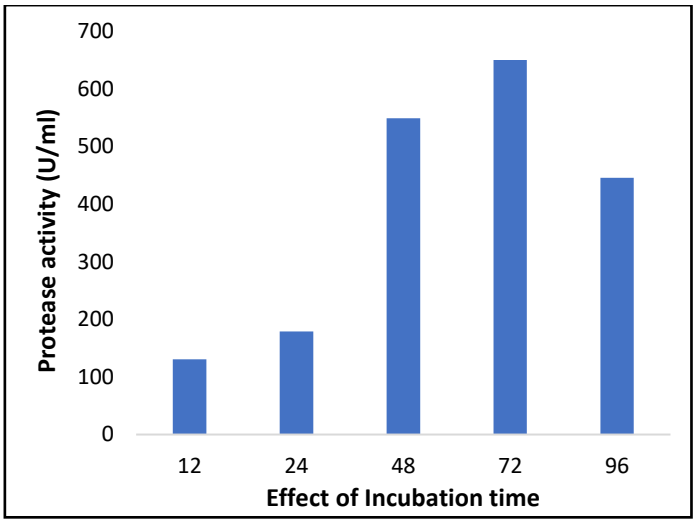


Figure 9 Effect of incubation time on protease activity

3.9 Extraction of Crude Enzyme at optimized condition

Under the optimized conditions determined through the OVAT approach—pH 7.0, temperature 40°C, and incubation time of 76 hours—the highest protease activity was observed in the selected isolate, Bacillus cereus, which exhibited an enzyme activity of 710.03 IU (Table 4.8). This result confirms that the optimized parameters significantly enhance protease production compared to other tested conditions (17). The high yield under these conditions validates the effectiveness of the OVAT method in identifying suitable environmental parameters for maximum enzyme production.

3.10 Purification of Protease Enzyme

The extracellular protease produced by Bacillus cereus was successfully purified using ammonium sulfate precipitation followed by dialysis. Precipitation at 85% ammonium sulfate saturation led to the concentration of the enzyme, which was then dissolved and dialyzed in 20 mM phosphate buffer (pH 6.5) to remove residual salts. After dialysis, the enzyme retained significant activity and was stored at –4°C for further use. The purification process resulted in a partially purified protease preparation suitable for downstream characterization (22). Although there was some loss in total protein content during dialysis, the specific activity of the enzyme was improved, indicating effective removal of non-enzymatic proteins and other interfering components.

3.11 Molecular characterization of Protease Producing Bacteria

The 16S rRNA gene of the bacterial isolate was successfully amplified using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). Agarose gel electrophoresis of the PCR product revealed a single, distinct band of approximately 1500 base pairs, confirming specific amplification of the target gene. The amplified fragment was purified and subjected to Sanger sequencing. The resulting sequence was analyzed using BLASTn against the NCBI database, which showed the highest similarity to Bacillus cereus ATCC 14579. To further confirm the taxonomic position, a phylogenetic tree was constructed using the Neighbor-Joining (NJ) method based on aligned 16S rRNA gene sequences of closely related Bacillus species (32). The query sequence clustered closely with Bacillus cereus ATCC 14579, supported by strong bootstrap values, indicating a close evolutionary relationship. These findings confirm the successful molecular identification of the isolate as a member of the Bacillus cereus group.

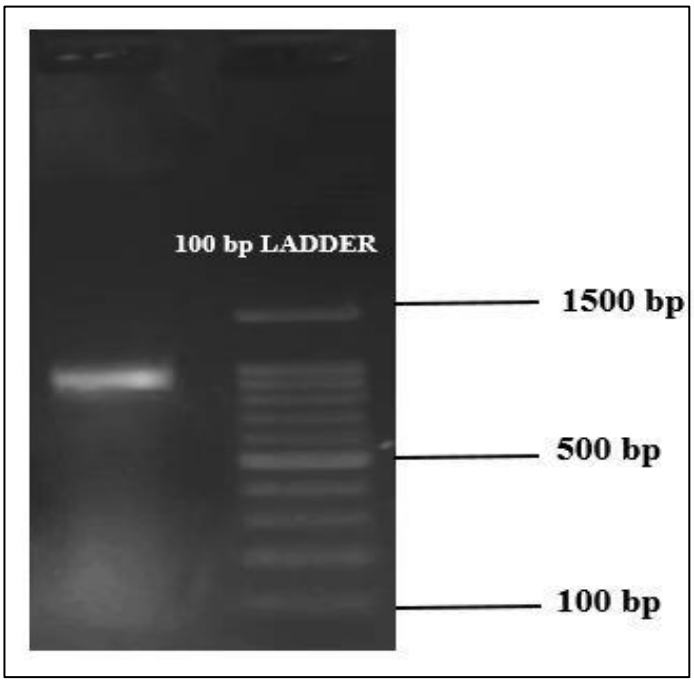


Figure. 10 Agarose gel electrophoresis of 16S rRNA gene PCR product

Lane 1: Amplified product (~1500 bp) from bacterial isolate using universal primers 27F and 1492R. Lane 2: 100 bp DNA ladder (molecular weight marker).

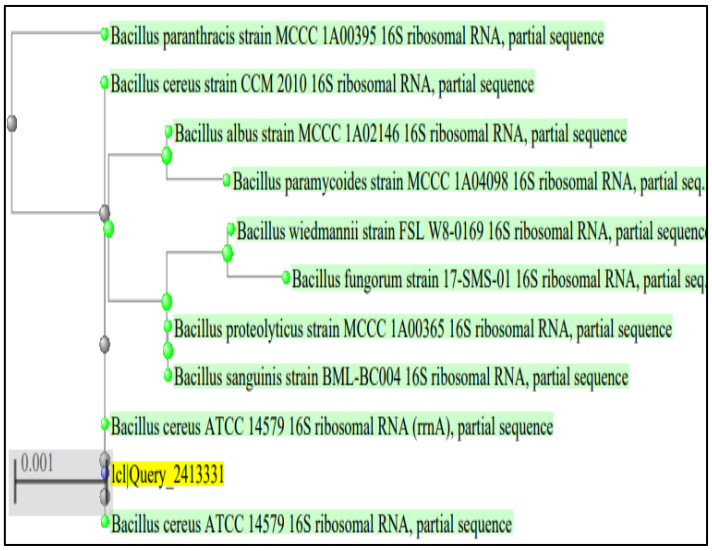


Figure.11 Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences

Figure.11 Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of the query sequence (highlighted in yellow) to closely related Bacillus species. The query clusters tightly with Bacillus cereus ATCC 14579, confirming its identification.

4. CONCLUSION

The present study successfully isolated and characterized protease-producing bacteria from the Kodungaiyur waste treatment zone, identifying a promising strain with potential pharmaceutical relevance. Among the isolates, A1 demonstrated the highest proteolytic activity (21 mm hydrolysis zone) and was identified as *Bacillus cereus* through biochemical characterization and 16S

rRNA sequencing. Optimization of fermentation parameters using the OVAT approach revealed maximum enzyme production at pH 7.0, 40°C, and 76 hours of incubation, yielding a peak activity of 710.03 U/mL. Partial purification through ammonium sulfate precipitation and dialysis enhanced the enzyme's specific activity, indicating improved functional efficiency. From a drug delivery perspective, the characterized alkaline protease exhibits significant potential as a biocatalyst in enzyme-responsive and controlled drug delivery systems. Its stability under varying physicochemical conditions supports its applicability in peptide-based therapeutics, targeted drug release, and the development of biodegradable delivery platforms. The enzyme's ability to function efficiently under near-physiological and mildly alkaline conditions further enhances its suitability for biomedical formulations. Future research should focus on integrating this protease into nano-enabled drug delivery systems, including polymeric nanoparticles, hydrogels, and enzyme-triggered release platforms. Detailed kinetic studies, structural characterization, and immobilization strategies will be essential to improve stability, reusability, and formulation compatibility. Additionally, recombinant expression and scale-up studies can enhance production efficiency, while in vitro and in vivo evaluations are necessary to validate its therapeutic performance and biosafety. Overall, this study provides a strong foundation for the development of marine-derived protease-based innovations in advanced drug delivery technology.

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AUTHOR CONTRIBUTIONS

Senthil Kumar Muniasamy: Conceptualization, Investigation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCE

[1] Afrin, S., Tamanna, T., Shahajadi, U.F., Bhowmik, B., Jui, A.H., Miah, M.A.S., Bhuiyan, M.N.I., 2024. Characterization of protease-producing bacteria from garden soil and antagonistic activity against pathogenic bacteria. The Microbe 4, 100123.

[2] Anandharaj, M., Sivasankari, B., Siddharthan, N., Rani, R.P.P., Sivakumar, S., 2016. Production, purification, and biochemical characterization of thermostable metallo-protease from novel Bacillus alkalitelluris TWI3 isolated from tannery waste. Applied Biochemistry and Biotechnology 178(8), 1666–1686.

[3] Anné, J., Economou, A., Bernaerts, K., 2016. Protein secretion in Gram-positive bacteria: From multiple pathways to biotechnology. In: Protein and Sugar Export and Assembly in Gram-Positive Bacteria, pp. 267–308.

[4] Baweja, M., Nain, L., Kawarabayasi, Y., Shukla, P., 2016. Current technological improvements in enzymes toward their biotechnological applications. Frontiers in Microbiology 7, 965.

[5] Elias, M., Wieczorek, G., Rosenne, S., Tawfik, D.S., 2014. The universality of enzymatic rate–temperature dependency. Trends in Biochemical Sciences 39(1), 1–7.

[6] Florencio, C., Couri, S., Farinas, C.S., 2012. Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by Trichoderma strains. Enzyme Research 2012, 793708.

[7] Homaei, A., Izadpanah Qeshmi, F., 2022. Purification and characterization of a robust thermostable protease isolated from Bacillus subtilis strain HR02 as an extremozyme. Journal of Applied Microbiology 133(5), 2779–2789.

[8] Ida, É.L., Silva, R.R., Oliveira, T.B., Souto, T.B., Leite, J.A., Rodrigues, A., Cabral, H., 2017. Biochemical properties and evaluation of washing performance in commercial detergent compatibility of two collagenolytic serine peptidases secreted by Aspergillus fischeri and Penicillium citrinum. Preparative Biochemistry and Biotechnology 47(3), 282–290.

[9] Imdakim, M.M., Hassan, Z., Aween, M.M., Elshaafi, I.M., Muhialdin, B.J., 2015. Milk clotting and proteolytic activity of enzyme preparation from Pediococcus acidilactici SH for dairy products. African Journal of Biotechnology 14(2), 133–142.

[10] Jensen, K., Ostergaard, P.R., Wilting, R., Lassen, S.F., 2010. Identification and characterization of a bacterial glutamic peptidase. BMC Biochemistry 11(1), 47.

[11] Kabense, R., Ginting, E.L., Wullur, S., Kawung, N.J., Losung, F., Tombokan, J.L., 2019. Screening of the proteolytic bacteria symbiont with algae Gracillaria sp. Jurnal Ilmiah PLATAX 7(2), 421–426.

[12] Kumar, D., Savitri, T.N., Verma, R., Bhalla, T.C., 2008. Microbial proteases and application as laundry detergent additive. Research Journal of Microbiology 3(12), 661–672.

[13] Nascimento, W.C.A., Martins, M.L.L., 2004. Production and properties of an extracellular protease from thermophilic Bacillus sp. Brazilian Journal of Microbiology 35, 91–96.

[14] De Oliveira, C.T., Rieger, T.J., Daroit, D.J., 2017. Catalytic properties and thermal stability of a crude protease from the keratinolytic Bacillus sp. CL33A. Biocatalysis and Agricultural Biotechnology 10, 270–277.

[15] Omidinia, E., 2012. Isolation, purification and characterization of a thermophilic alkaline protease from Bacillus subtilis BP-36. Journal of Sciences, Islamic Republic of Iran 23(1), 7–13.

[16] Qureshi, A.S., Khushk, I., Ali, C.H., Chisti, Y., Ahmad, A., Majeed, H., 2016. Coproduction of protease and amylase by thermophilic Bacillus sp. BBXS-2 using open solid-state fermentation of lignocellulosic biomass. Biocatalysis and Agricultural Biotechnology 8, 146–151.

[17] Rao, K., Narasu, M.L., 2007. Alkaline protease from Bacillus firmus 7728. African Journal of Biotechnology 6(21).

[18] Rehman, R., Ahmed, M., Siddique, A., Hasan, F., Hameed, A., Jamal, A., 2017. Catalytic role of thermostable metalloproteases from Bacillus subtilis KT004404 as dehairing and destaining agent. Applied Biochemistry and Biotechnology 181(1), 434–450.

[19] Umayaparvathi, S., Meenakshi, S., Arumugam, M., Balasubramanian, T., 2013. Purification and characterization of protease from Bacillus cereus SU12 isolated from oyster Saccostrea cucullata. African Journal of Biotechnology 12(40).

[20] Vijayaraghavan, P., Lazarus, S., Vincent, S.G.P., 2014. De-hairing protease production by an isolated Bacillus cereus strain AT under solid-state fermentation using cow dung: Biosynthesis and properties. Saudi Journal of Biological Sciences 21(1), 27–34.

[21] Beg, Q.K., Sahai, V., Gupta, R., 2003. Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor. Process Biochemistry 38, 1–7.

[22] Bundela, V., Mandal, S.K., 2013. Purification and characterization of an extracellular alkaline protease produced from an isolated Bacillus subtilis. International Journal of Applied Biology and Pharmaceutical Technology 4, 112–119.

[23] Sevinc, N., Demirkan, E., 2011. Production of protease by Bacillus sp. N-40 isolated from soil and its enzymatic properties. Journal of Biological and Environmental Sciences 5, 95–103.

[24] Prakasham, R.S., Rao, C.S., Sharma, P.N., 2006. Green gram husk – an inexpensive substrate for alkaline protease production by Bacillus sp. in solid-state fermentation. Bioresource Technology 97, 1449–1454.

[25] Olajuyigbe, F.M., Ajele, J.O., 2005. Production dynamics of extracellular protease from Bacillus species. African Journal of Biotechnology 4, 776–779.

[26] Kumar, C.G., Takagi, H., 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnology Advances 17, 561–594.

[27] Jisha, V.N., Smitha, R.B., Pradeep, S., Sreedevi, S., Unni, K.N., Sajith, S., Priji, P., Josh, M.S., Benjamin, S., 2013. Versatility of microbial proteases. Advances in Enzyme Research 1, 39–51.

[28] Gupta, R., Beg, Q.K., Khan, S., Chauhan, B., 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Applied Microbiology and Biotechnology 60, 381–395.

[29] Aretz, W., Koller, K.P., Riess, G., 1989. Proteolytic enzymes from recombinant Streptomyces lividans TK24. Biochemical Engineering Journal 65, 31–36.

[30] Bascaran, V., Hardisson, C., Brana, A.F., 1990. Regulation of extracellular protease production in Streptomyces clavuligerus. FEMS Microbiology Letters 34, 208–213.

[31] Boyer, H.W., Carlton, 1968. Production of two proteolytic enzymes by transformable strain of Bacillus subtilis. Archives of Biochemistry and Biophysics Research Communications 128, 442–455.

[32] Cherdyntseva, T.A., Razin'kov, V.K., Egorov, N.S., 1982. Bacillus subtilis var. amyloliquefaciens biosynthesis of extracellular protease possessing coagulase activity and formed under conditions of limiting the nitrogen sources in the medium. Mikrobiologiia 51, 431–435.

[33] Jeong, K.C., Jeong, H.S., Rhee, J.H., Lee, S.E., Chung, S.S., Starks, A.M., Escudero, G.M., Gulig, P.A., Choi, S.H., 2000. Construction and phenotypic evaluation of a Vibrio vulnificus vvpE mutant for elastolytic protease. Brazilian Journal of Microbiology 68, 5096–5106.

[34] AbdelGalil, O.A., 1992. Fermentation of proteases by Aspergillus fumigatus and Penicillium sp. Journal of King Saud University 4(2), 127–136.

[35] Bhaskar, N., Sudeepa, E.S., Rashmi, H.N., Selvi, A.T., 2007. Partial purification and characterization of protease of Bacillus proteolyticus CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresource Technology 98, 2758–2764.

[36] Brown, E.D., Yada, R.Y., 1991. Spin-labelling and differential scanning calorimetry study of the denaturation of aspartic proteinase from the fungi Endothia parasitica and Mucor miehei. Agricultural and Biological Chemistry 55, 1639–1641.

[37] Buchanan, R.E., Gibbons, N.E., 1974. Bergey's Manual of Determinative Bacteriology. 8th ed. Baltimore: The Williams & Wilkins Co., pp. 15–36.

[38] Chekireb, D., Tahar, A., Cochet, N., 2009. Acid protease production by isolated species of Penicillium. European Journal of Scientific Research 25(3), 469–477.

[39] Deng, A., Wu, J., Zhang, Y., Zhang, G., Wen, T., 2010. Purification and characterization of a surfactant-stable high-alkaline protease from Bacillus sp. B001. Bioresource Technology 101, 7100–7116.