

Production, Purification, and Characterization of an Industrially Important Enzyme Alkaline Protease Produced from Locally Isolated *Bacillus* Bacteria

Ranjeet Singh*, Ravindra Meena, Harsh Kumar, Banwari Lal

Environmental and Industrial Biotechnology Division, The Energy and Resources Institute, New Delhi, India

Email address:

ranjeet.singh@teri.res.in (R. Singh)

*Corresponding author

To cite this article:

Ranjeet Singh, Ravindra Meena, Harsh Kumar, Banwari Lal. Production, Purification, and Characterization of an Industrially Important Enzyme Alkaline Protease Produced from Locally Isolated *Bacillus* Bacteria. *International Journal of Microbiology and Biotechnology*. Vol. 6, No. 4, 2021, pp. 137-146. doi: 10.11648/j.ijmb.20210604.15

Received: November 9, 2021; **Accepted:** December 9, 2021; **Published:** December 24, 2021

Abstract: The worldwide demand for industrially essential enzymes is growing due to their eco-friendly and sustainable applications in broad industrial sectors. The main objective of this investigation is intended to isolate potent alkalophilic native bacteria from local habitats for the cost-effective production of an enzyme alkaline protease. For screening of alkalophilic bacteria, bacterial samples were taken from various natural alkaline habitats, and bacterial cultures were screened on agar plate having skimmed milk as protein substrate by using the protein hydrolysis method. The bacteria showing maximum proteolytic potential were identified by microscopic, biochemical, and 16S rDNA analysis. Additionally, the culture components and other medium parameters have been optimized for higher enzyme production. High yield alkaline protease was obtained with conditions of 1% inoculum, pH 9.0, and at 37°C temperature, with 1% sugarcane molasses as the best suitable carbon substrate. Partial purification of the enzyme was performed and characterized for their optimum activity on various parameters (temperatures and pH range). The approx. molecular weight of the enzyme was estimated to be ~35 kDa. This study demonstrates the production of an industrially important enzyme (alkaline protease) from a newly isolated native *Bacillus* spp. Economical production of this enzyme can be commercially applied in vast industrial sectors like agriculture, textile, food, detergent, and leather industries.

Keywords: Agro-industrial Residues, Alkaline Protease, *Bacillus* Spp, Cost-effective, Sugarcane Molasses

1. Introduction

Recently, the interest in eco-friendly technologies has been growing rapidly to mitigate pollution at the industrial level [1]. Being an environment-friendly product, enzymes are attributed to having many sustainable applications in various industries [2]. Proteases are critical biological enzymes present in all living organisms for essential metabolic reactions, physiological processes, and cellular growth [3]. Protease enzymes are the most prominent members among all enzyme classes owing to specific characteristics desired to be wide industrial usages [4]. Protease enzyme encompasses about 60-70% of the worldwide enzyme shares from biological sources [5]. Alkaline proteases from bacterial sources are considered to be the most suitable and potentially

more significant compared with animal, fungi, and plant protease because of their characteristics of being high proteolytic activity at alkaline pH range (pH 7-10), broad substrate specificity [6] and for ease of performing genetic manipulations to alter the enzyme properties. [7]

Moreover, the optimal temperature of bacterial alkaline protease is around 50°C-60°C making them highly suitable for applications in different industrial sectors [8]. Alkaline protease enzymes are isolated from diverse genera of bacteria, including *Bacillus* [4] *Streptomyces* [9, 10], *Thermoactinomyces* [11, 12], *Nesterenkonia* [13], *Brachystreptospora* [14], and *Saccharopolyspora* [15]. However, among the various bacteria, *Bacillus* spp. are found to be the major source of alkaline protease enzyme under extremes of conditions [16] using simple carbon sources.

Alkaline proteases from *Bacillus* spp. are reported to be stable and active over a range of pH and temp. with lower purification costs [17]. Nowadays, there is a growing demand in industries for innovative and cost-efficient methods for enhancing the production of alkaline proteases to make them more commercially successful [18].

Further, as it is well established that the cost for fermentative production of any enzyme is greatly dependent on components of the growth medium, especially nitrogen and carbon substrates [19], as well as culture parameters, e.g., substrate concentration, pH, temperature, incubation period [20, 21]. Therefore, to develop the production process more industrially viable, the medium components and cultural parameters must be optimized to fulfill the industrial demands [22]. Since alkalophilic bacteria isolated from local natural habitat could be a significantly cheaper source of an enzyme as they are easily isolated and maintained and these bacteria are generally more resistant to hostile conditions, which make them economical for commercial applications. Therefore, in this study, our aim was screening and isolation of indigenous soil alkalophilic bacterial species to isolate the most potent producer, and then standardized the culture parameters for high yield production of the target enzyme alkaline protease and its characterizations. Further, different renewable agro-industrial residues as cheap carbon sources have been explored for cost-effective medium formulation for enzyme production. The optimized production process will contribute a sustainable means for agro-industrial residues utilization as well as minimization in the cost of substrate for alkaline protease production to meet the various industrial demands for this industrially important enzyme.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

For isolation of alkalophilic bacteria having potential for alkaline enzyme production, soil samples from different environmental conditions were aseptically isolated from diverse locations in and around the Gualpahari region in Haryana, and all samples were processed immediately in the laboratory for screening. The appropriate dilutions of samples were checked for their proteolytic activity by plating onto a medium containing nutrient-agar and skim milk maintained at an alkaline pH (1% skim milk, 2% agar, 0.5% NaCl, and 0.1% peptone, pH 10.0). The bacterial cultures were kept at 37°C in a controlled environment for up to 48 hrs, and zones of clearance around the colonies of bacteria were observed, which indicates proteolytic activity by the bacterial colonies. The cultures were further microscopically identified and differentiated based on the morphology, culture conditions, and other biochemical properties. The purity of the selected cultures was checked using Gram's staining. All the selected bacterial strains were maintained at 4°C in nutrient agar tubes for further experiments.

2.2. Isolation and Identification of Bacterial Strains

The pure cultures of the selected bacteria were grown and further screened for reproducibility of alkaline protease production by streaking and spotting them on Luria-Bertani (LB) agar plate containing skim milk (pH 10.0). The production of enzymes by the screened bacteria was confirmed by reproducible development of a hydrolysis zone around the bacterial colonies onto the plate. A total of 50 alkalophilic bacteria were isolated for the protease activity, where 10 isolates were confirmed as potent producers of alkaline protease. Out of all the 10 bacterial isolates, *Bacillus* spp. RS3 showed the maximum clearance zone with respect to the other isolated strains; hence, the isolate RS3 has been picked up for further studies. All the selected bacterial strains were characterized by 16S rDNA based sequencing approach, where the pure culture was allowed to grow at 37°C in LB broth for up to 24-48 h and then the culture was harvested, lysed, and genomic DNA was isolated from the bacteria using cell lysis method. Further, taking genomic DNA as a template, 16S rDNA was PCR amplified by Thermocycler (Eppendorf Mastercycler® nexus gradient) using the universal primer pairs specific for the 16S rDNA sequence (primer 1: 5'ATCGGATCCATGAGAAGCAAAAAATTGTGG 3', primer 2 5' CTCGAATTCTTATTGTGCAGCTGCTTGTA 3'). Then the amplified 16S rDNA products were purified by agarose gel purification method, and a purified DNA sample was sent for sequencing through an automated sequencer (Macrogen incorporation, Korea). The 16S rDNA sequence obtained was analyzed for similarity using the online available BLAST search engine. The isolated bacteria were identified by multiple sequence alignment through nBLAST analysis [23], where the similar sequences obtained from the BLAST result were aligned using the default settings of the CLUSTAW program (Mega 6, 2013 version). The phylogenetic tree analysis of the sequences was done by the Neighbor-Joining method [24]. All the cultures have been stored in TERI's culture collection depository with the reference ID *Bacillus* spp. RS3.

2.3. Alkaline Protease Production

The selected culture positive for the higher alkaline protease The selected cultures positive for the higher protease secretion were grown in a standard medium (Glucose 5%, Peptone 7.5%, NaCl-5%, FeSO₄·7H₂O-0.1%, MgSO₄·7H₂O-5%, pH 10.0), under the optimal condition at 37°C [25]. The medium was aerobically grown at 37°C for up to 60 hours in a shaker incubator (120-200 rpm) and monitored for maximum alkaline protease production. After different time intervals of growth, the cultures were pelleted at 4°C by centrifugation at 7000 rpm for a minimum of 10 min to recover the active crude extracellular enzyme. The culture supernatant containing the crude protease was taken periodically for further enzyme assays and characterization studies.

2.4. Characterization of Protease Enzyme and Protease Activity Assay

All the samples were processed as described earlier, and the total protein content of each sample was estimated by adopting the Bradford method [26], where culture supernatants were mixed with Bradford reagent in a 1:5 ratio, respectively. Then the tubes were gently mixed, and after incubation, the absorbance readings were taken at 595nm in a UV-Visible spectrophotometer (Shimadzu, USA). The protein concentrations were estimated by taking BSA as standard proteins concentrations. The enzyme activity assays were performed as reported by McDonald and Chen [27]. Briefly, crude extracts were mixed with casein (1% w/v) dissolved in a standard buffer (0.1N Glycine-NaOH, pH 10) and kept for incubation at 60°C for 10-15 min. Then stop solution (10% Trichloroacetic acid) was added to terminate the reaction, and then mixtures were centrifuged at 10,000 rpm for 10 min. Then the filtrates were added with Folin-ciocalteau reagent and kept for incubation for about half an hour. Then reading was taken at 700 nm. 1 unit of enzyme activity was calculated as the quantity of protease needed to liberate 1 µg of tyrosine per ml per min. Tyrosine concentrations in the range of 50-250 µg were taken as standard concentrations for calculation.

2.5. Culture Parameters Optimization for Alkaline Protease Production

For optimum production of the enzyme, the influence of different growth factors on enzyme production was assessed by growing the bacteria in a basal medium under various parameters like growth in different carbon sources, nitrogen sources, temperature, pH, time of incubation, and substrates. To this end, the effect of temperature on alkaline protease production was analyzed on broth culture grown at various temperatures, i.e., from 20°C-70 °C including an interval of 10°C for 24 hr at pH 10.0. The culture supernatants were assayed for enzyme activity analysis. For time-course studies, the culture was allowed to grow for different incubation times in hours (3, 6, 12, 24, 36, 48, 54, and 60) and their effect on the enzyme production was studied. The culture supernatants were isolated at the respective time, and enzyme activity was measured. Further, the optimum pH for high yield of enzyme production has been analyzed by growing the bacteria in a basal medium having different pH (3, 4, 5, 6, 7, 8, 9, 10, 11, and 12) using required concentrations of 1N NaOH.

2.6. Media Optimization of Different Carbon Sources and Nitrogen Components

Identification of best carbon and nitrogen sources for high yield production of the enzyme, the glucose from the standard medium was replaced with different agricultural residues as sole carbon sources such as rice husk (RH), lignocellulose waste (LCW), starch waste (SW), sugarcane molasses (SM) and sugarcane bagasse (SB) by adopting one variable at one time approach. Where the suitable carbon

source was identified by assaying the culture supernatant after incubation for enzyme activity with total protein content under standard assay conditions. Similarly, to optimize the nitrogen source, seven different sources of nitrogen such as peptone, yeast extract, ammonium chloride (AC), beef extract, ammonium nitrate (AN), and ammonium sulphate (AS) were mixed to basal medium and bacterial culture was incubated for 48 hours.

2.7. Partial Purification of the Target Enzyme

For partial purification of the target enzyme from crude preparation, the culture supernatant (crude protease) was collected at 4°C by centrifugation at 7000 rpm to get the cell-free filtrate. About 500 ml of the cell-free filtrate was first brought to a saturation level of 20% (w/v) with ammonium sulphate and then up to 100% saturated. Then the samples were dialyzed against phosphate buffer at pH 7.0 in a dialysis bag (cut off 12kDa) for 24 hours with constant stirring and intermittent buffer change. The isolated protease enzyme preparation was concentrated and stored at 4°C. All the purified fractions were analyzed as described earlier, and a purity check of the isolated enzyme was performed by SDS-PAGE methods.

3. Results

3.1. Morphological Identification and Characterization of Bacterial Strain

Alkalophilic bacterial cultures from different soil samples were screened for enzyme activity. Out of 50 isolates, 10 isolates were isolated as potent producers of alkaline protease by observing a zone of hydrolysis around the bacterial colonies onto an agar plate containing skim milk protein as substrate (Figure 1). Among the isolated 10 strains, *Bacillus* spp. RS3 showed the maximum zone of clearance and alkaline protease production in comparison with other strains, and it has been chosen for future experimental analysis (Table 1). Further, based on the morphological study, biochemical characterization, and phylogenetic tree analysis with default settings of CLUSTAW program (Mega 6, 2013 version) of 16S rDNA sequence of the isolate RS3, it is observed that the strain RS3 has the maximum similarity (100%) with *Bacillus megaterium* strains (Figure 8). The complete 1418 bp sequence of 16S rDNA has been deposited to the database (NCBI) with accession no. MH660377.

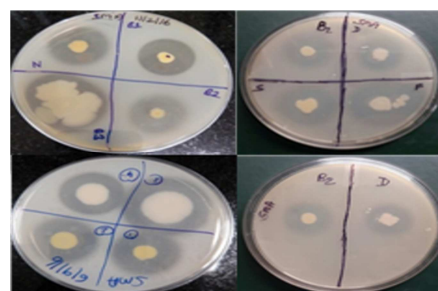


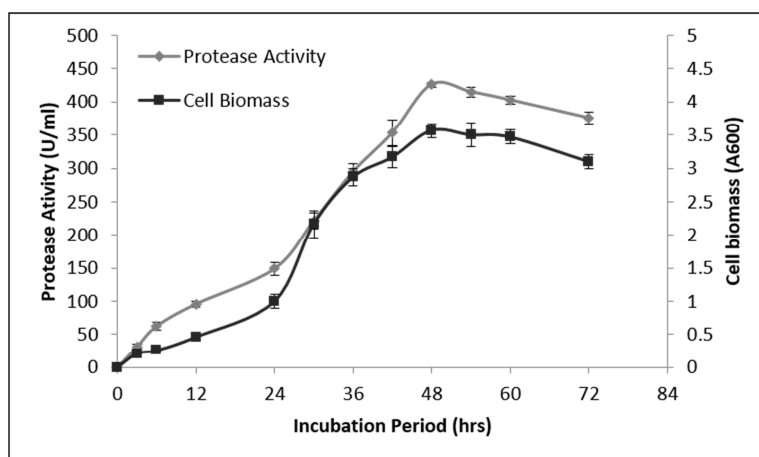
Figure 1. Production of alkaline protease by alkalophilic bacteria on an agar plate with skim milk as substrate.

Table 1. Alkaline protease production by different isolates.

Isolates	Zone diameter (mm)	Protease activity (U/ml)
RS1	16	240
RS2	26	310
RS3	28	330
RS4	21	290
RS5	21	295
RS6	12	200
RS7	22	280
RS8	19	270
RS9	17	265
RS10	23	300

3.2. Optimization of Growth Period for Alkaline Protease Production

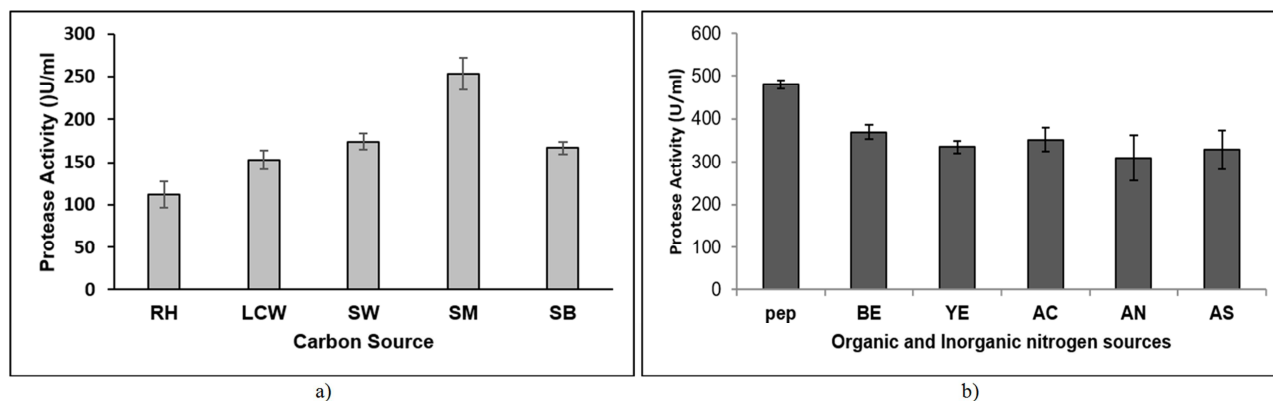
The effect of growth period on isolate RS3 has been observed as a gradual increment on the yield of the enzyme from starting to up to 48 hours and maximum production was found to be at 48 hours with the enzyme activity of 426 U/ml (Figure 2). [28], have reported similar results in *Bacillus* odyssey with up to 72 h of incubation time. Under identical conditions, the effect of different incubation periods on enzyme production has also been reported by other groups e.g., 36 h in *Streptomyces* sp. CN902 [29] and 60 h for subtilisin production by *Bacillus* bacteria sp. [30].

**Figure 2.** Growth and alkaline protease yield by *Bacillus* strain. RS3.

3.3. Optimization of Culture Substrate on Enzyme Production

The effect of various substrates (carbon and nitrogen) on the production of enzyme was assessed by growing bacteria in the basal medium having various agri-industrial residues as sole carbon sources such as rice husk (RH), lignocellulose waste (LCW), starch waste (SW), sugarcane molasses (SM) and sugarcane bagasse (SB) at various concentrations. Out of the five different substrates used for the growth of alkaline protease production, sugarcane molasses was observed to be

the superior substrate with a high titer of protease activity, as evident from Figure 3a. Afterward, the bacterial strains have been grown in a formulated fermentation media containing sugarcane molasses as the chief carbon source with other media nutrients as described earlier. Similarly, the growth media has also been optimized with various nitrogen sources, e.g., Peptone (Pep), Beef Extract (BE), Yeast Extract (YE), Ammonium chloride (AC), Ammonium Nitrate (AN), and Ammonium sulphate (AS), and the highest production was obtained with 1% peptone (Figure 3b).

**Figure 3.** Effect of different substrates for alkaline protease yield by *Bacillus* strain RS3. 3a) effect of different carbon sources on protease production, 3b) Effect of various nitrogen sources (inorganic and organic) on alkaline protease yield. The bars represent the standard deviation of experimental replicates studied.

3.4. Optimization of Substrate and Inoculum Concentrations

Since the amount of substrate can contribute significantly to the enzyme yield; therefore, firstly, the effect of substrate concentrations (sugarcane molasses) on the alkaline protease production was analyzed. To this end, the highest enzyme production was observed when 1% substrate was taken in our experimental condition. Further increasing the concentrations substrate in the flask decreased the enzyme yield (Figure 4a). Hence, 1% sugarcane molasses is selected as the optimum

concentration for further experiments. Furthermore, the influence of the concentration of substrate inoculum on enzyme yield by isolate RS3 was also determined by growing the bacteria for 48 at 37°C with different concentrations of inoculum sizes (pH 9.0). Where, higher yield with lower inoculum size (1%) has been observed, and beyond 1%, there was a reduction in the enzyme production (Figure, 4b). Hence, 1% inoculum size has been selected as the optimum concentration of inoculum for further experiments.

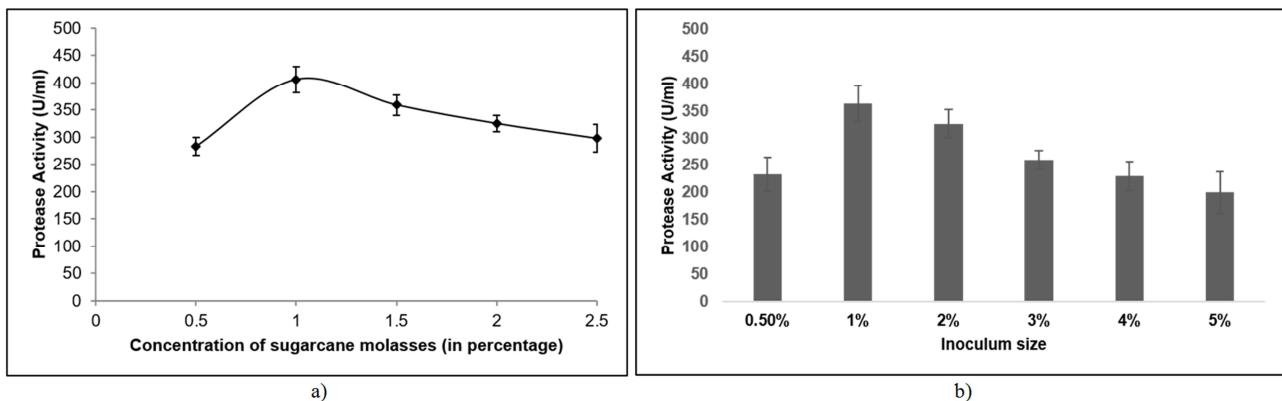


Figure 4. Influence of substrate concentration and inoculum size on alkaline protease yield. 4a) Effect of different concentrations of sugarcane molasses, and 4b) Effect of different inoculum concentrations.

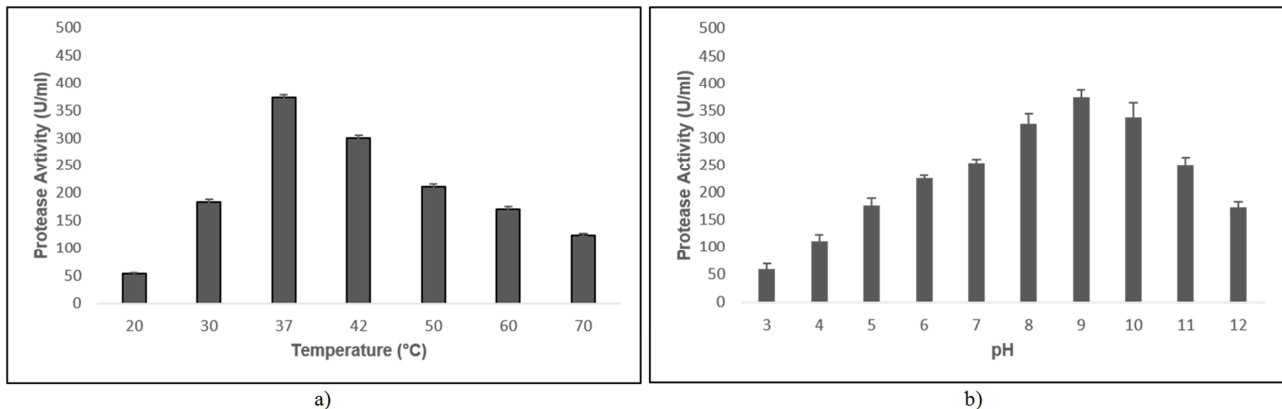


Figure 5. Standardization of culture parameters on alkaline protease production by isolate RS3. 5a) Influence of different temperatures on enzyme yield. 5b) Influence of different pH on enzyme production in isolate RS3.

3.5. Optimization of Culture Conditions on Enzyme Yield

The optimization of various culture conditions on the enzyme yield was studied by incubating the bacteria in basal media where sugarcane molasses was taken as carbon substrate, and for nitrogen, source peptone was utilized. Further, the optimizations were done with respect to other parameters like temperature and pH. Here, the first investigation was done on the influence of different temperatures on the enzyme yield by growing the culture at various incubation temperatures in the range of 20°C to 70°C at 150 rpm, where the incubation time remained

constant at 50 hrs (Figure 5a). In all the procedures, samples were analyzed for respective time intervals for protease activity. Similarly, the yield against the range of culture pH (pH range of 3-12) on protease production was also analyzed with pH in using required concentrations of 1N NaOH (Figure 5b).

3.6. Enzyme Activity Assay for Alkaline Protease

The activity assay of alkaline protease was carried out with 1% casein dissolved in a standard buffer (Glycine-NaOH, pH 10.0). The reaction mixture was then kept at 37°C for up to 0.5 hr. Then 10% stop solution

(trichloroacetic acid) was mixed to terminate the process. The tyrosine liberated during the reaction was estimated using a Folin-ciocalteu reagent by measuring the absorbance reading at OD₆₇₀. A standard range of 50-250 µg of tyrosine concentration was used for calculation. Where 1U of enzyme activity is determined as per the standard formula.

3.7. Characterization of Crude Enzyme

For the characterization of alkaline protease, the culture supernatant (crude enzyme) from broth culture of *Bacillus* spp. RS3 was partially purified and subjected to preliminary characterization studies where the influence of two parameters (temperature and pH) on enzyme stability and activity were studied.

3.8. Partial Purification of Alkaline Protease from Crude Extract

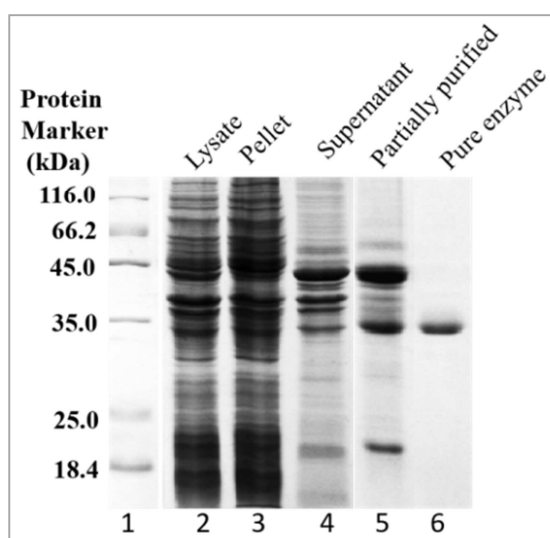


Figure 6. SDS-PAGE analysis of partially purified alkaline protease. Lane 1: Protein Marker; Lane 2: Whole bacterial lysate; Lane 3: cell pellet; Lane 4: Culture supernatant; Lane 5: Ammonium sulphate precipitated partially purified protein; Lane 6: purified alkaline protease.

The partial purification of the enzyme was done by precipitations with solid ammonium sulphate, where culture supernatant was mixed with 30 to 80% (w/v) ammonium sulphate precipitation then centrifuged at min for precipitation, then the collected pellet was suspended and dialyzed against phosphate buffer (pH 7.0) and kept at 4°C. The activity and molecular mass of the enzyme were estimated by SDS-PAGE analysis. As shown in Figure 6, an approximately 35 kDa molecular weight band was observed as the purified alkaline protease corresponding with the prediction made from the gene sequence confirming that the purified protein is the alkaline protease.

3.9. Effect of Temperature and pH on Alkaline Protease Activity

The optimum temperature and thermal stability of the protease activity were examined by taking the enzyme preparation in phosphate buffer, including casein as enzyme substrate. The reaction was incubated under standard assay conditions at different temperatures (20°C to 80°C) for 1 hour. (Figure 7a). To this end, the optimal temperature for the highest activity of the enzyme was observed around 37°C. To examine the thermal stability, the enzymatic reaction was incubated further for 30 min in the same conditions, and the activity was monitored. The enzyme maintained its basal activity from 25°C to 50°C but rapidly lost its activity at high temperatures, and activity was eventually down to 65°C. Similarly, the effect of pH on the catalytic activity of the enzyme was determined by incubating the reaction mixture under standard assay conditions with a wide pH range (3 to 12) using the different buffer solutions (KCl-HCl-pH 2.0, Citrate buffer-pH 3-6, phosphate-pH 7), Tris-HCl buffer-pH 8-9 and Glycine-NaOH buffer-pH 10-12. Where the enzyme exhibited maximum activity around pH 9.0 (Figure 7b). The stability profile of the purified enzyme was also determined by measuring the residual activity at a broad pH value at 4°C and found that the enzyme was stable between pH 7.0 to 10.0 where it retained more than 90% of its activity.

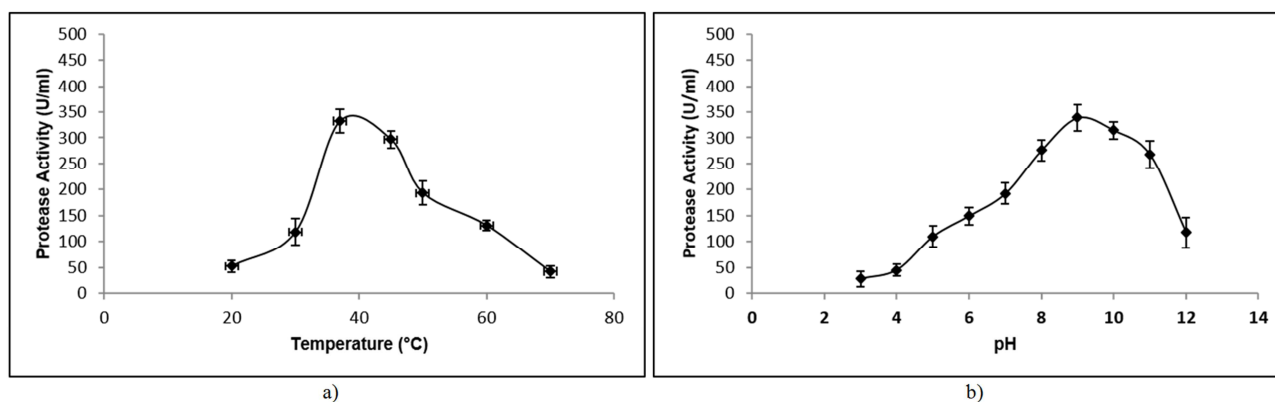


Figure 7. Effect of temperature and pH on the activity of partially purified alkaline protease isolated from *Bacillus* spp. RS3 6a) protease activity at different temperatures and b) Protease activity at different pH.



Figure 8. Phylogenetic tree of isolate RS3 showing the relationship of isolate RS3 to other *Bacillus* spp. with their accession numbers. Each number on a branch indicates the bootstrap values.

4. Discussion

Alkaline proteases are a class of protease enzymes with immense commercial significance owing to their eco-friendly applications in various industries. In the present study, samples were collected to isolate indigenous alkalophilic bacteria for high yield production of an industrially important enzyme alkaline protease and its biochemical characteristics. A total of 50 indigenous *Bacillus* spp. of bacteria from different local soil samples were collected and analyzed for their ability to produce extracellular protease enzymes. Out of these 50 protease-producing isolates, 10 isolates were identified as potent producers of alkaline protease based on the size of clear zone around the colonies on the screening media plate having skimmed milk agar as described by Jani et al., 2016 [31]. Out of the 10 selected isolates, one isolate (RS3) showed the highest alkaline protease production ability with respect to all other isolates tested on the plate. The isolate RS3 was further optimized for high alkaline protease production at different culture parameters like incubation period and different cell densities in a standard medium, and maximum production was found to be at 48 h with the protease activity of 426 U/ml. Similar observations were reported in a *Bacillus tequilensis* strain [32] and in a *Bacillus coagulans* strain [33] where 48 hours of incubation period was noted to be an optimum period for maximum yield of the enzyme.

Further increase of incubation time led to the gradual decrease in enzyme production, suggesting enzyme production is associated with the bacteria's growth and plays a primary metabolic role during the log phase of bacterial growth for utilization of nutrients present in the substrate [34, 35]. Further, we developed a cheap medium formulation for isolate RS3 using the renewable agri-industrial residues where the culture medium was supplemented with different renewable carbon and nitrogen substrates for bacterial growth. Interestingly, sugarcane molasses and peptone showed the highest protease production by the isolate RS3. Also, the production of enzymes was observed to be gradually increasing with increasing concentration of

sugarcane molasses from 0.5% to up to 1%, and after that, there was no further increase in enzyme production, indicating that optimum concentrations of sugarcane molasses have a significant effect on the growth of bacteria and the yield of the desired product. In an analogous study, the equivalent influence of sugarcane molasses on protease yield was noted in *B. subtilis* [36]. The 1% concentration of sugarcane molasses is observed to be optimum may possibly be due to the availability of enough quantity of nutrients in the medium for bacterial growth along with the maximum secretion of alkaline protease. Many studies have reported that organic nitrogenous substrate have significant effect on protease production in comparison to inorganic nitrogen compounds [37]. These observations are consistent with a similar study where maximum protease production was reported with peptone as a sole nitrogen component in the medium [38]. Similar findings were also observed in *B. stearothermophilus* F1 where the yield of thermostable protease was compared with organic and inorganic nitrogen compounds [39]. Further, the optimum conditions such as growth temperature and time, culture medium pH, and inoculum size on alkaline protease yield were also determined to be at 37°C, 48 hours, 9 and 1% respectively as previously reported in *Bacillus licheniformis* P003 [40]. The short incubation period is considered to be favorable for the cost-effective production of an enzyme, and the optimal pH was in agreement with some previous reports in various *Bacillus* sp. where the pH for maximum protease secretion was between 7.0 to 10.0 [41, 42, 43]. The reduced enzyme production beyond 1% inoculum size may be due to reduced dissolved oxygen in the medium and increased competition for nutrients [44].

Further, preliminary characterization studies with partially purified enzyme showed that the enzyme is thermostable with optimum activity at 37°C and pH 9.0 with a reaction mixture containing casein (1%) as substrate and incubated for 1 hour period. The observation is consistent with a similar study in *Bacillus licheniformis* B18 [45]. The apparent molecular weight of the partially purified enzyme was estimated to be ~35 kDa, as shown on SDS-PAGE. This molecular mass was in accordance with

previous reports, where the molecular mass of alkaline proteases from various *Bacillus* sp. of bacteria was reported to be less than 40 kDa. [46, 47]. Further, the enzyme had optimal activities over a broad pH range (8-11) and exhibited a temperature optimum of 60°C [48]. An analogous study also reported similar stability of protease enzyme in broad pH range (pH 6-11) in *Bacillus licheniformis* MH31 [49]. Since pH of the culture broth also affects the stability of the enzyme being produced. Therefore, bacteria were allowed to grow in the medium of different pH (pH range of 3-12), and protease production was analyzed. To this end, maximum enzyme production was noted in the medium of pH 9.0 as reported in similar studies with other *Bacillus* spp. [50, 51]. Molecular identification of isolate RS3 was also carried out by 16S rDNA sequencing method using the NCBI's online BLAST search tool, and the complete nucleotide sequence has been deposited to the NCBI database with accession no. MH660377. The study, therefore, concludes that the promising features of the isolated *Bacillus* spp. RS3 strain for production of alkaline protease enzyme shall increase the potential use of the isolated strain as a biotechnological tool to meet the demands of various industrial applications and activities.

5. Conclusion

In the present study, an indigenous alkaline protease-producing alkalophilic bacteria was isolated from local soil samples as a potent producer of alkaline protease enzyme. The new isolate was identified as *Bacillus* spp. RS3 based on biochemical tests and molecular identification using 16S rRNA sequence analysis on NCBI's online BLAST search tool. For the cost-effective production of the alkaline protease by *Bacillus* spp. RS3 agricultural residues were used as cheaper substrates than the conventional substrates. The fermentation parameters for *Bacillus* spp. RS3 were optimized in order to increase the production of extracellular alkaline protease with high activity. The effect of various renewable substrates as carbon and nitrogen sources on enzyme production was assessed where a combination of 1% (w/v) sugarcane molasses as carbon source and 1% (w/v) peptone as nitrogen source were observed to be superior substrates with a higher titer of protease activity. The other culture conditions such as optimum incubation time, temperature, inoculum size, and pH of the medium for the maximum enzyme production were determined as 48 hours, 37°C, 1% and pH 9.0, respectively. Preliminary characterization of the partially purified enzyme showed that the enzyme is thermostable up to 50°C with optimum activity at 37°C and pH 9.0, which indicate characteristic features of alkaline proteases. The molecular weight of the partially purified alkaline protease was estimated to be ~35 kDa. Further, the enzyme had optimal activities over a broad pH range (8-11), and a temperature optimum of 60°C. The isolated *Bacillus* spp. RS3 strain and the partially purified enzyme may further be

exploited for various potential industrial applications such as in the leather industry, laundry detergent, dairy production, and the pharmaceutical industry.

Conflict of Interest

Authors declare no conflict of interest.

Acknowledgements

The authors are grateful to Director, Environment and Industrial Biotechnology Division, The Energy and Resources Institute, New Delhi, India, for providing the necessary research facilities to complete this study.

References

- [1] C. Zhou, H. Qin, X. Chen, Y. Zhang, Y. Xue, and Y. Ma, "A novel alkaline protease from alkaliphilic *Idiomarina* sp. C9-1 with potential application for eco-friendly enzymatic dehairing in the leather industry," *Sci. Rep.*, no. May, pp. 1–18, 2018.
- [2] M. D. Pastor, G. S. Lorda, A. Balatti, S. Rosa, and L. Pampa, "PROTEASE OBTENTION USING *BACILLUS SUBTILIS* 3411 AND AMARANTH SEED MEAL MEDIUM AT DIFFERENT AERATION RATES," pp. 6–9, 2001.
- [3] A. Burhan, U. Nisa, C. Gökhan, C. Ömer, A. Ashabil, and G. Osman, "Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6," *Process Biochem.*, 2003.
- [4] R. Gupta, Q. Beg, and P. Lorenz, "Bacterial alkaline proteases: Molecular approaches and industrial applications," *Appl. Microbiol. Biotechnol.*, vol. 59, no. 1, pp. 15–32, 2002.
- [5] N. Annamalai, M. V. Rajeswari, and T. Balasubramanian, "Extraction, purification and application of thermostable and halostable alkaline protease from *Bacillus alveayuensis* CAS 5 using marine wastes," *Food Bioprod. Process.*, 2014.
- [6] V. Jisha *et al.*, "Versatility of microbial proteases," *Adv. Enzym. Res.*, vol. 01, no. 03, pp. 39–51, 2013, doi: 10.4236/aer.2013.13005.
- [7] S. Lin, M. Zhang, J. Liu, and G. S. Jones, "Construction and application of recombinant strain for the production of an alkaline protease from *Bacillus licheniformis*," *J. Biosci. Bioeng.*, vol. 119, no. 3, pp. 284–288, 2015.
- [8] K. Adinarayana, P. Ellaiah, and D. S. Prasad, "Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11," *AAPS PharmSciTech*, vol. 4, no. 4, p. E56, 2003.
- [9] L. A. I. De Azeredo, L. R. Castilho, S. G. F. Leite, R. R. R. Coelho, and D. M. G. Freire, "Protease production by *Streptomyces* sp. isolated from Brazilian Cerrado soil: Optimization of culture medium employing statistical experimental design," 2003.
- [10] K. Kathiresan and S. Manivannan, "Production of alkaline protease by *Streptomyces* sp., isolated from coastal mangrove sediment," *Res. J. Environ. Sci.*, 2007.

- [11] S. Ç. Aksoy, A. Uzel, and E. E. Hameş Kocabaş, "Extracellular serine proteases produced by *Thermoactinomyces* strains from hot springs and soils of West Anatolia," *Ann. Microbiol.*, 2012.
- [12] D. H. Petrova, S. A. Shishkov, and S. S. Vlahov, "Novel thermostable serine collagenase from *Thermoactinomyces* sp. 21E: Purification and some properties," *J. Basic Microbiol.*, 2006.
- [13] S. Bakhtiar, R. J. Estiveira, and R. Hatti-Kaul, "Substrate specificity of alkaline protease from alkaliphilic feather-degrading *Nesterenkonia* sp. AL20," *Enzyme Microb. Technol.*, 2005.
- [14] S. D. Gohel and S. P. Singh, "Characteristics and thermodynamics of a thermostable protease from a salt-tolerant alkaliphilic actinomycete," *Int. J. Biol. Macromol.*, 2013.
- [15] G. Raut, S. Vetal, R. Biao, X. Y. Liu, L. Zhang, and C. Kokare, "Purification and characterization of organic solvent and detergent stable protease isolated from marine *Saccharopolyspora* sp. A9: Application of protease for wound healing," *Biochem. Eng. J.*, 2012.
- [16] M. Schallmeyer, A. Singh, and O. P. Ward, "Developments in the use of *Bacillus* species for industrial production," *Can. J. Microbiol.*, vol. 50, no. 1, pp. 1–17, 2004.
- [17] K. Jellouli *et al.*, "Molecular and biochemical characterization of an extracellular serine-protease from *Vibrio metschnikovii* J1," *J. Ind. Microbiol. Biotechnol.*, 2009.
- [18] J. S. Mukherjee *et al.*, "Programmes and principles in treatment of multidrug-resistant tuberculosis," *Lancet*, vol. 363, pp. 474–481, 2004.
- [19] B. K. Lakshmi, P. Ratna sri, K. Ambika Devi, and K. Hemalatha, "Screening, optimization of production and partial characterization of alkaline protease from haloalkaliphilic *Bacillus* sp," *Int. J. Res. Eng. Technol.*, vol. 3, no. 2, pp. 435–443, 2014.
- [20] R. N. Z. R. A. Rahman, M. Basri, and A. B. Salleh, "Thermostable alkaline protease from *Bacillus stearothermophilus* F1; nutritional factors affecting protease production," *Ann. Microbiol.*, 2003.
- [21] N. A. Razak, M. Y. A. Samad, M. Basri, W. M. Z. W. Yunus, K. Ampon, and A. B. Salleh, "Thermostable extracellular protease of *Bacillus stearothermophilus*: factors affecting its production," *World J. Microbiol. Biotechnol.*, 1994.
- [22] B. Bhunia, B. Basak, and A. Dey, "A review on production of serine alkaline protease by *Bacillus* spp.," *J. Biochem. Tech.*, vol. 3, no. 4, pp. 448–457, 2012.
- [23] K. Saeki *et al.*, "A novel species of alkaliphilic *Bacillus* that produces an oxidatively stable alkaline serine protease," *Extremophiles*, vol. 6, no. 1, pp. 65–72, 2002.
- [24] N. Saitou and M. Nei, "The neighbor-joining method: a new method for reconstructing phylogenetic trees," *Mol. Biol. Evol.*, vol. 4, no. 4, pp. 406–425, 2017.
- [25] K. Krishnaveni, D. J. Mukesh Kumar, M. D. Balakumaran, S. Ramesh, and P. T. Kalaichelvan, "Production and optimization of extracellular Alkaline Protease from *Bacillus subtilis* isolated from dairy effluent," *Der Pharm. Lett.*, vol. 4, no. 1, pp. 98–109, 2012.
- [26] M. M. Bradford, "Bradford assay for protein quantification," *Anal. Biochem.*, 1976.
- [27] C. E. McDonald and L. L. Chen, "The Lowry modification of the Folin reagent for determination of proteinase activity," *Anal. Biochem.*, 1965.
- [28] S. D. Tambekar and D. H. Tambekar, "Optimization of the production and partial characterization of an extracellular alkaline protease from thermo-halo-alkalophilic Lonar lake bacteria," *Biosci. Discov.*, vol. 4, no. 1, pp. 30–38, 2013.
- [29] V. Bhaskar *et al.*, "Optimization of production of subtilisin in solid substrate fermentation using response surface methodology," *J. Biotechnol.*, vol. 7, no. 13, pp. 2286–2291, 2008.
- [30] C. N. Aguilar, G. Gutiérrez-sánchez, A. Plilia, R. Rodríguez-herrera, J. L. Martínez-hernandez, and J. C. Contreras-esquivel, "No Title," vol. 4, no. 4, pp. 354–366, 2008.
- [31] S. A. Jani, Y. M. Parekh, T. N. Parmar, and T. J. Dalwadi, 'Screening and Characterization of Alkaline Protease Producing *Bacillus* Strain B-4 *Bacillus flexus* and Study of its Potential for Alkaline Protease Production', vol. 5, no. 5, pp. 767–787, 2016.
- [32] K. Aruna, J. Shah, and R. Birmole, 'Production and Partial Characterization of Alkaline Protease from *Bacillus Tequilensis* Strains Csgab0139 Isolated from Spoilt Cottage Cheese', *Int. J. Appl. Biol. an Pharm. Technol.*, vol. 5, no. 3, pp. 201–221, 2014.
- [33] F. M. Olajuyigbe, 'Optimized production and properties of thermostable alkaline protease from *Bacillus subtilis* SHS-04 grown on groundnut (*Arachis hypogaea*) meal', *Adv. Enzym. Res.*, vol. 01, no. 04, pp. 112–120, 2013.
- [34] S. A. Ul Qadar, E. Shireen, S. Iqbal, and A. Anwar, 'Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3', *Indian J. Biotechnol.*, vol. 8, no. 3, pp. 286–290, 2009.
- [35] A. Sumantha, C. Larroche, and A. Pandey, 'Microbiology and industrial biotechnology of food-grade proteases: A perspective', *Food Technol. Biotechnol.*, vol. 44, no. 2, pp. 211–220, 2006.
- [36] A. S. Qureshi, M. A. Bhutto, I. Khushk, and M. U. Dahot, 'Optimization of cultural conditions for protease production by *Bacillus subtilis* EFRL 01', *African J. Biotechnol.*, vol. 10, no. 26, pp. 5173–5181, 2011.
- [37] 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.*, vol. 97, no. 13, pp. 1449–1454, 2006.
- [38] K. Ash, A. Milton Lall, K. Rao, and P. W. Ramteke, 'Production and Optimization of an Alkaline Protease from *Acinetobacter variabilis* Isolated from Soil Samples', *Int. J. Agric. Environ. Biotechnol. Cit. IJAEB*, vol. 11, no. 2, pp. 379–386, 2018.
- [39] R. N. Z. R. A. Rahman, M. Basri, and A. B. Salleh, 'Thermostable alkaline protease from *Bacillus stearothermophilus* F1; nutritional factors affecting protease production', *Ann. Microbiol.*, 2003.
- [40] 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, 2013.

- [41] S. Kaur, R. M. Vohra, M. Kapoor, Q. K. Beg, and G. S. Hoondal, 'Enhanced production and characterization of a highly thermostable alkaline protease from *Bacillus* sp. P-2', *World J. Microbiol. Biotechnol.*, vol. 17, no. 2, pp. 125–129, 2001.
- [42] S. Puri, Q. K. Beg, and R. Gupta, 'Optimization of alkaline protease production from *Bacillus* sp. by Response surface methodology', *Curr. Microbiol.*, vol. 44, no. 4, pp. 286–290, 2002.
- [43] C. G. G. Kumar, 'Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*', *Lett. Appl. Microbiol.*, vol. 34, no. 1, pp. 13–17, 2002.
- [44] I. Saadoun, R. Rawashdeh, T. Dayeh, Q. Ababneh, and A. Mahasneh, 'Isolation, characterization and screening for fiber hydrolytic enzymes-producing streptomycetes of Jordanian forest soils', *Biotechnology*, vol. 6, no. 1, pp. 120–128, 2007.
- [45] G. Lakshmi and N. N. Prasad, 'Purification and Characterization of Alkaline Protease from a Mutant *Bacillus licheniformis* BI8', vol. 9, no. 1, pp. 15–23, 2015.
- [46] J. Singh, N. Batra, and R. Sobti, 'Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1', *Process Biochem.*, vol. 36, no. 8, pp. 781–785, 2001.
- [47] D. young Yum, H. C. Chun, D. H. Bai, and D. H. Oh, 'Purification and Characterization of Alkaline Serine Protease from an Alkalophilic *Streptomyces* sp', *Biosci. Biotechnol. Biochem.*, vol. 58, no. 3, pp. 470–474, 1994.
- [48] P. Sarker, S. Talukdar, P. Deb, S. Sayem, and K. Mohsina, 'Optimization and partial characterization of culture conditions for the production of alkaline protease from *Bacillus licheniformis* P003', *Springerplus*, vol. 2, no. 1, p. 506, 2013.
- [49] Yu, Jeong-Hyeon & Jin, Hyun-Seok & Choi, Woo-Young & Yoon, Min-Ho, 'Production and Characterization of an Alkaline Protease from *Bacillus licheniformis* MH31', *J. Appl Biol Chem.*, vol. 49, no. 4, pp. 135-139, 2006.
- [50] P. Sathyavathan and S. Krithika, 'Production and Optimization of Protease from *Bacillus licheniformis* NRRL-NRS-1264 using cheap source substrates by submerged (SmF) and solid-state fermentation (SSF)', *Int. J. ChemTech Res.*, vol. 6, no. 1, pp. 286–292, 2014.
- [51] W. C. A. Do Nascimento and M. L. Leal Martins, 'Production and properties of an extracellular protease from thermophilic *Bacillus* sp', *Brazilian J. Microbiol.*, vol. 35, no. 1–2, pp. 91–96, 2004.
- [52] F. Pouryafar, G. D. Najafpour, N. Noshadi, and M. Jahanshahi, 'Thermostable alkaline protease production via solid state fermentation in a tray bioreactor using *Bacillus licheniformis* ATCC 21424', *Int. J. Environ. Res.*, vol. 9, no. 4, pp. 1127–1134, 2015.