Research Article
Hittite Journal of Science and Engineering, 2023, 10 (2) 135–144
ISSN NUMBER: 2148–4171
DOI: 10.17350/HJSE19030000300

Production, Optimization and Partial Characterization of Alkaline Protease from *Bacillus subtilis* spp. subtilis NRRL B-3384 and B-3387

Cengiz Akkale
Adana Alparslan Türkeş Bilim ve Teknoloji University, Department of Bioengineering, Adana, Türkiye

Article History:
Received: 2023/01/17
Accepted: 2023/03/17
Online: 2023/06/30

***Correspondence to**: Cengiz Akkale,*
E-Mail: cengizakkale@gmail.com
Phone: +90 (322) 455 0000
Fax: +90 (322) 455 0002

This article has been checked for similarity.

ABSTRACT

Bacillus subtilis has been a reliable platform for the expression of extracellular proteases for several decades. Although a majority of *Bacillus subtilis* subspecies express proteases, the amount of secreted enzyme varies depending on the strain and environmental conditions used. Here, two *Bacillus subtilis* spp. subtilis strains, NRRL B-3384 and NRRL B-3387, from the ARS Culture collection (NRRL), were compared for secreted protease activity. The highest activity was found in strain NRRL B-3384, and proteolysis occurred at temperatures as high as 80°C and across a broad range of pH, with maximum activity at pH 9.0 and 60°C indicating the presence of a thermostable alkaline protease. To our knowledge, this is the first study to evaluate protease production in *Bacillus subtilis* spp. subtilis strains NRRL B-3384 and B3387 and suggests that NRRL B-3384 may have utility in the production of enzymes for industrial use.

Keywords:
Bacillus subtilis, *Protease*, Enzyme, Activity

INTRODUCTION

Commercial enzymes facilitate efficient use of raw materials, reduce the need for toxic chemicals and minimize waste in a wide variety of industrial processes (1, 2). Proteases (EC 3.4.21), in particular, are in widespread use (3, 4) with numerous applications in the food, detergent, pharmaceutical, leather, textile and agricultural industries (5). These enzymes catalyze the hydrolysis of the peptide bonds in proteins and polypeptides (6) and are classified based on the position of the peptide bond they cleave and the pH range at which they are most active (7, 8).

By and large, the production of proteases relies on microbial fermentation. Bacterial and fungal systems are simple and inexpensive to grow, and their enzymes often outperform those in animal and plant systems. The genus, *Bacillus*, a group of Gram-positive, rod-shaped, spore-forming bacteria, is perhaps the most commonly used organism for protease production (3, 9). Indeed, at least ten species of *Bacillus*, including *B. subtilis* (10), *B, luteus* (11), *B. clausii* (12), *B. pumilus* (13, 14), *B, mojavensis* (15), *B. infantis* (16), *B. koreensis* (17), B. sphaericus (18), B. firmus (19) and B. licheniformis (20, 21) have been harnessed for this purpose.

From the standpoint of production, *Bacillus* spp. are adapted to a wide range of growth conditions (22), and are generally inexpensive to grow, safe to handle, and easy to manipulate at the genetic level. Numerous studies have been done to enhance production via strain selection (23), methods of cultivation (2), media composition (11), and reaction conditions (24). Furthermore, some of the proteases identified in this genus have been found to have remarkable properties such as high stability in extreme conditions, biodegradability and high yield (9).

With the idea that large publicly accessible culture collections could be mined for useful isolates, this study aims to examine previously unstudied *Bacillus* subspecies or strains for robust expression of proteolytic enzymes with novel properties. Partial characterization of protease activity from two such strains from the ARS Culture Collection, *Bacillus subtilis* spp. subtilis NRRL B-3384 and B-3387, is described here.

Cite as:
Akkale C,. Production, optimization and partial characterization of alkaline protease from bacillus subtilis spp. subtilis NRRL B-3384 and B-3387. Hittite J Sci Eng 2023;10(2):135-144. doi:10.17350/hjse19030000300

MATERIAL AND METHODS

Bacterial strains

Bacillus subtilis spp. *subtilis* strains (NRRL B-3384 and NRRL B-3387) were obtained from ARS Culture Collection (NRRL) repository in Peoria, IL.

Screening for protease production

Overnight liquid bacterial cultures were diluted 1:100 in 5 ml of TGY (Tryptone-Glucose-Yeast Extract) media (tryptone 0.5%, yeast extract 0.5%, K_2HPO_4 0.1%, glucose 0.1%, pH 7.0) in a sterile 50 ml culture tube and grown for six hours at 30°C with orbital shaking at 180 rpm to obtain logarithmic growth phase cultures. One µl of log-phase liquid culture was then inoculated directly onto the middle of a selective media plate (peptone 0.5%, yeast extract 0.5%, K_2HPO_4 0.1%, glucose 0.1%, agar 1.5%) at two different pH levels (pH=6, pH=8) containing either casein, skim milk powder or gelatin from bovine skin (1%) as a protein substrate for proteolytic activity. After liquid was adsorbed onto the agar, plates were incubated at three different temperatures (24°C, 30°C, 37°C) for 24 h. The release of protease enzyme was determined by presence of a clear zone around individual colonies on the selective media. Ratios of clear zone to colony diameter were used as a semi-quantitative measurement of protease production.

Effect of culture media pH on bacterial growth and protease production

Cell growth and protease production of the strain NRRL B-3384 were studied at four pH levels (5, 6, 7 and 8). The inoculum was prepared by growing the bacterial culture in TGY media. Optimum pH was determined by growing cultures in 250 ml baffled flasks containing 50 ml buffered media (0.5% tryptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.1% glucose, 1% milk powder, pH 5-8) at 30°C for 70 hours in a shaker incubator (200 rpm). Optical density at 600 nm (OD600) was used for determining growth and the protease activity was measured as per assay procedure.

Effect of temperature, protein source, and incubation time on growth and protease activity

To analyze effects of large protein sources, liquid cultures were prepared in baffled flasks containing media with 1% (w/v) of selected protein. Skim milk powder, casein, and gelatin were compared as protein sources in cultures grown at either 20°C or 30°C for 54 h at 200 rpm agitation. In the first 24 h, samples were taken at 3 h intervals to measure growth and protease activity. Subsequent samples were taken at different time intervals ranging from 2 h to 18 h.

Fed-batch production in lab scale bioreactor

Fed-batch fermentation was conducted in a 2.5 L stirred tank bioreactor (MiniBio 2500, Applikon Biotechnology, Delft, Netherlands) at 800 ml working volume with temperature and pH control. An inoculum was prepared by transferring 10 µl of frozen stock of *Bacillus subtilis* strain NRRL B-3384 into 100 ml of optimized medium (0.5% tryptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.1% glucose, 1% milk powder) in a 500 ml baffled flask and incubating at 30°C with orbital shaking at 200 rpm for 16 h. The inoculum was added to the bioreactor containing sterilized optimized medium at a ratio of 1:7. Fermentation was carried out for 48 h at 30°C. Dissolved oxygen was maintained at 30% via a cascaded control of agitation rate (from 300 rpm to 1000 rpm) depending on O_2 demand. The aeration rate was maintained at 0.25 vvm. The pH was set to 8 and controlled with addition of 1 M HCl solution. Foam was controlled by manual addition of antifoam (Antifoam Y-30 Emulsion, Sigma-Aldrich) at 0.5 ml/L. For fed-batch cultivation, 100 ml 8% milk powder solution was added at 12 and 36 hours of cultivation. Production broth was periodically sampled for analysis of cell growth and protease activity. Experiments were performed in duplicate and mean values are presented. At the end of production, whole fermentation broth was centrifuged at 3000 × g for 30 min and the supernatant was stored at 4°C for further analysis, modified from Çoban (25).

Protease activity measurement and optimization

Protease activity was determined using the protocol previously described by Çoban (25) and Vaithanomsat et al. (26) with some modifications. Culture medium was centrifuged at 3000 x g for 10 min. Reaction mixture containing 0.5 ml of casein solution (0.6% (w/v) casein in 50 mM phosphate buffer (pH=8)) and 0.1 ml of culture supernatant was incubated at 35°C. Different incubation times (t=5, t=10, t=15, t=20, t=30, t=40 min) were tested to find optimum incubation time. The reaction was stopped by adding 0.5 ml 0.44 M trichloroacetic acid solution, incubated for 10 minutes at room temperature, and then centrifuged at 3000 x g for 10 minutes.

A 0.25 ml aliquot from the resulting supernatant was mixed with 1.25 ml Na_2CO_3 (0.5 M) and 0.25 ml Folin phenol Ciocalteau reagent (1 N) followed by incubation for 20 minutes at 40°C. The absorbance of the mixture was measured at 680 nm against distilled water as the blank. Respec-

tive sterile media was used in place of culture supernatant as a negative control. Absorbances obtained from negative controls were subtracted from absorbances obtained from test samples to calculate protease activities. A tyrosine standard curve was obtained using tyrosine solutions ranging from 0 to 100 mg/L. Solutions were mixed with carbonate buffer and Folin phenol Ciocalteau reagent and incubated and measured as described above. One unit of protease activity was defined as the amount of enzyme which liberated 1 µg of tyrosine per minute at 35°C. The equation below was used for the calculation of protease activity of the samples in U/ml;

$$\text{Protease activity (U/ml)} = \frac{(A_{sample} - A_{negative\ control}) \times \text{Reaction volume (ml)} \times \text{Dilution factor}}{\text{Slope of standart line} \times \text{Duration of reaction (min)} \times \text{Enzyme volume (ml)}} \quad (1)$$

where A_{sample} and $A_{negative\ control}$ are absorbances of protease assay mixtures of samples taken from various experimental conditions and their corresponding sterile media, respectively.

Effect of temperature, pH, and divalent cations on protease activity

To determine the effect of temperature on enzyme activity, protease reactions were carried out at temperatures ranging from 10°C to 90°C. The effect of pH on enzyme activity was determined by using buffers at pH levels from 5 to 10.6 (acetate buffer for pH 5, phosphate buffer for pH 6, 7 and 8, carbonate buffer for pH 9, 10 and 10.6). Changes in protease activity were also analyzed following the addition of various metals ($MgSO_4$, $MnCl_2$, $CaCl_2$, $ZnSO_4$, $CuSO_4$, EDTA) to the reaction mixture to a final concentration of 20 mM. Standard assay conditions were implemented as described above, except that the reaction time was increased to 10 min.

Statistical analysis

Significance of difference in bacterial growth and protease activity among different experimental setups were analyzed by one way ANOVA (Analysis of Variance) and Tukey HSD (honestly significant difference) tests.

RESULTS

Comparison of protease activity by different strains using selective plate methods

Relative protease activities in *Bacillus subtilis* spp. *subtilis* strains NRRL B-3384 and NRRL B-3387 were determined at either pH 6 or pH 8 using semi-quantitative agar plate assays in which the ratios of clear zone:colony diameter were measured on different protein substrates, casein, milk powder or gelatin (Figure 1).

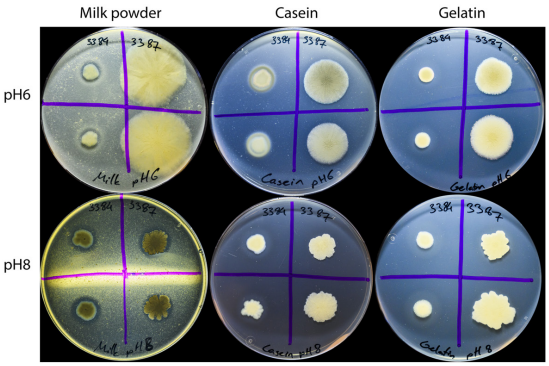

Figure 1. Clear zone surrounding colonies of NRRL B-334 and NRRL B-337 showing protease activity on milk powder, casein and gelatin containing agar plates (24 h incubation, pH 8.0). and pH 6.0

As shown in Table 1, of the two strains examined, NRRL B-3384 showed the highest activity on all three substrates and at both levels of pH. Overall, the highest ratio of clear zone to colony diameter (2.01:1) was obtained at pH 8.0 with gelatin as a protein source. When incubated at 20°C, a higher ratio was seen using milk powder as the substrate, although at the same temperature, discernible zones of clearance were not visible with either gelatin or casein (data not shown). At higher temperature (37°C), plates were difficult to read due to the overgrowth of colonies into zones of clearance (not shown). Based on these findings, the NRRL B-3384 strain was selected for further optimization experiments in this study based on clear zone to colony size ratios.

Table 1. Colony and clear zone diameters obtained from selective plate experiments using different protein sources at two pH levels and incubated at 30°C.

Strain	Protein source	pH	R: Clear zone diameter (mm)	r: Colony diameter (mm)	Ratio: R/r
B-NRRL 3384	Milk powder	6	11.8	8.5	1.38
		8	15.4	10.9	1.41
	Casein	6	14.5	8.8	1.64
		8	17.2	10.8	1.59
	Gelatin	6	14.8	8.8	1.67
		8	19.6	9.8	2.01
B-NRRL 3387	Milk powder	6	39.6	39.0	1.01
		8	17.7	14.2	1.25
	Casein	6	24.6	24.2	1.02
		8	17.2	17.1	1.01
	Gelatin	6	0.0	22.8	0.00
		8	20.2	18.6	1.08

Effect of pH on the growth and protease activity

To investigate the effect of initial pH on the production of protease, bacteria were grown at four different pH levels for 70 h. Milk powder was used as protein source based

on the results from agar plates. Maximum cell growth was observed at pH 6 while highest protease activity was obtained at pH 8 (Figure 2a and 2b).

Since the highest protease activity was at pH 8 in this study, subsequent submerged culture experiments were all conducted at this pH.

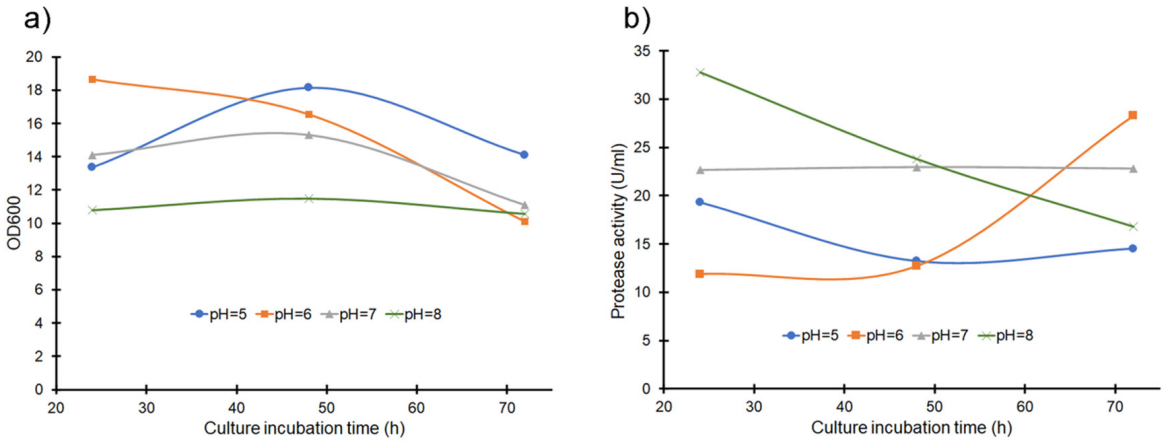

Figure 2. Effect of pH on the culture growth (a) and protease activity (b).

Effect of temperature, various protein sources, and incubation time on the growth and protease activity

A series of experiments was performed to study the effect of different temperatures and protein sources on the protease activity. Similar clear zone areas were obtained at the 20°C and 30°C incubation temperature on the agar plates in the present study, production of protease was also carried out at 20°C in the submerged culture to investigate the effect of low temperature on production of protease. In 250 ml baffled flask cultures, optimum temperature was 30°C for both protease activity and cell growth with all protein sources used (Figure 3). The maximum recorded protease activity by the strain NRRL B-3384 was 96.42 U/mL when incubated at 30°C and casein was used as a protein source. When it was incubated at 20°C with same protein source, maximum protease activity was 84.18 U/ml.

Among various protein sources used for protease production, maximum protease activity (96.42 U/mL) was achieved with casein supplement at 30°C followed by milk powder (68.8 U/ml) at 30°C. Casein and milk powder showed higher protease activity (84.2 U/ml and 63.6 U/ml, respectively) compared to gelatin when the incubation was carried out at 20°C. Furthermore, in the case of gelatin, no significant differences were seen in protease activity at 20°C versus 30°C (23.2 U/ml and 22.3 U/ml, respectively).

Incubation duration for culture growth and protease activity by strain NRRL B-3384 is shown in Figure 3. Different protein sources and incubation temperatures resulted in different culture growth and protease activity profiles (Figure 3a and 3b). Formation of protease started at early logarithmic phase and reached its near maximum in 12 h (90.8 U/ml) with casein at 30°C after which it slowed down and was stable until mid-stationary phase at 30 h.

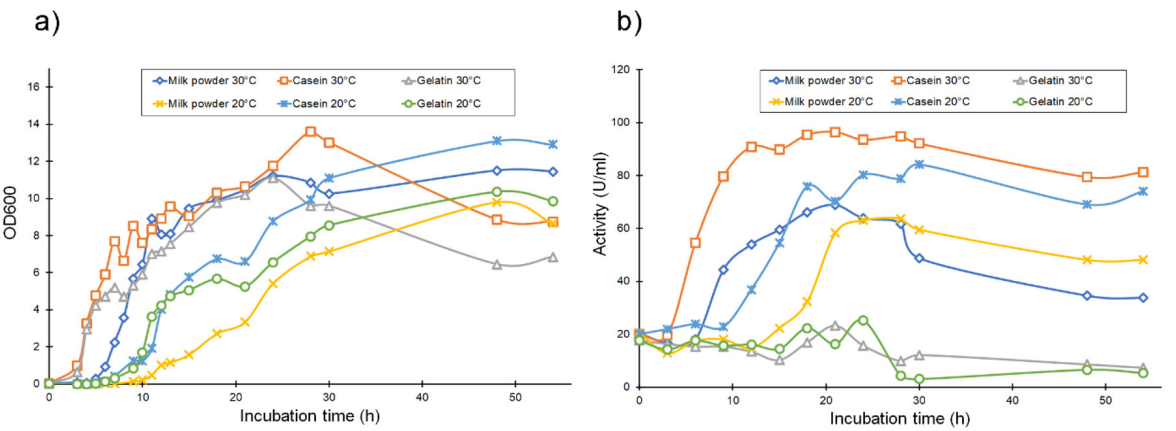

Figure 3. Effect of temperature and different protein sources on culture growth (a) and protease activity (b).

One way ANOVA done using protease activity levels from t= 21 h to 30 h shown in Figure 3b revealed the difference was significant ($\alpha<0.05$). Pairwise comparisons of experimental sets by Tukey HSD showed experimental sets with milk powder and casein had significantly different protease activity levels compared to sets with gelatin.

Protease production in lab scale bioreactor

In this study, it is aimed to evaluate strain NRRL B-3384 from this perspective by conducting a preliminary production in a 2.5 L bioreactor with an 800 ml working volume. Maximum OD of 13 in the bioreactor was reached 13 hours after inoculation (Figure 4). Maximum protease activity was 72.3 U/ml at 48th hour.

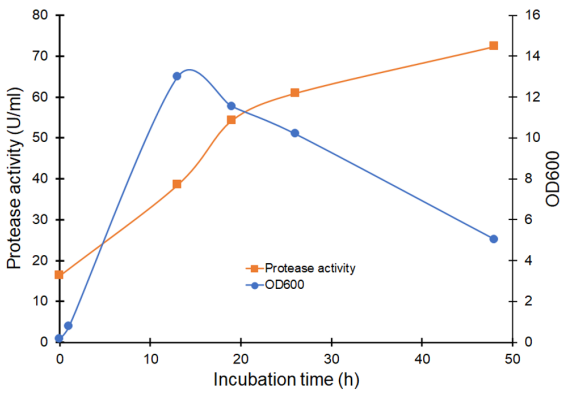

Figure 4. Effect of incubation time on the growth and protease production of the strain NRRL B-3384 in bioreactor scale production.

Effect of protease assay duration on protease activity measurement

To optimize the incubation time with substrate mixture (0.6% (w/v) casein in 50 mM phosphate buffer (pH=8)), protease and substrate were incubated for different time periods (5-40 min) at 35°C. The optimum enzyme activity was obtained at 5 min of incubation time as indicated in Figure 5.

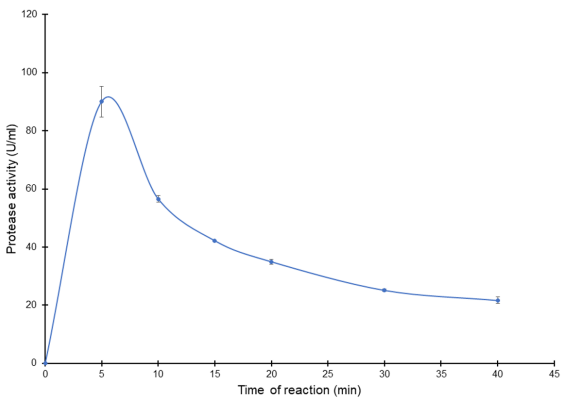

Figure 5. Protease activity vs time of protease reaction.

Effect of temperature, pH, and divalent cations on protease activity

Protease activity was analyzed at different temperatures ranging from 10°C to 90°C at pH of 8. In this study, maximum protease activity was found to be 60°C and activity decreased at higher temperatures (Figure 6a). However, approximately 75% of proteolytic activity retained at 80°C which is valuable for high temperature applications such as detergent and textile industry.

The crude enzyme also showed a uniform activity across a wide range of pH levels (5 to 11) (Figure 6b). While the optimal pH was found to be 10 it was also at least 90% active in both slightly acidic and strong alkaline conditions.

In this study, while $MgSO_4$, $CaCl_2$ and $ZnSO_4$ did not significantly affect the activity, $CuSO_4$ and EDTA increased the activity by 2.0 and 2.4-fold, respectively (Figure 6c). $MnCl_2$ made the highest impact on the enzyme and increased the activity 7.5-fold.

DISCUSSION

Effect of growth conditions, nutrients, and culture time on protease expression

In search of the most efficient producers of protease enzyme among *Bacillus* species, comparative experiments have been carried out using different protein sources. Clear zone formation around colonies in selective agar plates have been the main method of choice for determining protease activity semi-quantitatively. Vaithanomsat et al. (26) reported a zone diameter of 18 mm from *Bacillus licheniformis* in casein-agar plate with a clear zone diameter to colony diameter ratio of 1.8 when they screened different strains for protease production. Gelatin plates were also used for screening of protease producers including halophilic *Bacillus* sp. isolated from soil (27) and extremophilic *Halobacterium* sp. Js1, isolated from thalassohaline environment (28). For screening of proteolytic activity in different *Bacillus* strains, milk powder agar media was also used by other researchers (29-32) and prominent clear zone around the colony was also reported similar to this study. Different from our study, Pant et al. (33) used small disks infused with culture supernatants on selective agar plates to semi-quantitatively determine proteolytic activity at different time points of bacterial growth. It can be argued that this method is labor intensive and can be replaced with protease activity assay for liquid cultures (26).

Khan et al. (34) reported that optimum pH for cell growth and protease activity was 7 and 10, respectively

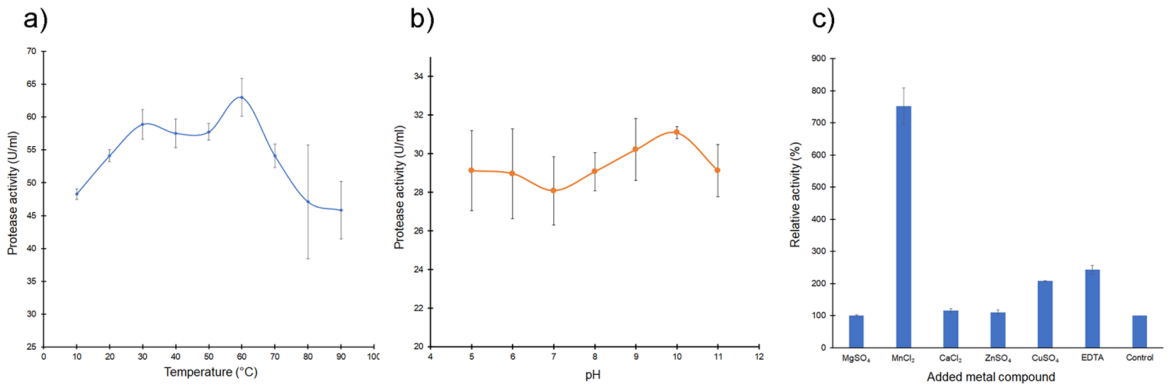

Figure 6. Effect of temperature (a), pH (b) and various compounds (c) on protease activity.

when they used *Bacillus tequilensis* strain MTCC 9585. Also, Ali et al. (29) showed that *Bacillus subtilis* strain BLK-1.5 showed the highest activity at pH 10 whereas the best growth was observed at pH 9. In accordance with these studies, our results showed no significant correlation between cell growth and protease activity.

Physical parameters such as temperature and incubation time can play an important role for expression of any enzyme, and they vary depending on the organisms (35). In the literature, temperatures ranging from 30°C to 65°C have been reported as optimal for the bacterial cell growth and production of protease from *Bacillus* strains (9).

The optimum temperature for protease production was 30°C when *Bacillus* sp. MIG strain was used (36). Asha et al. (37) and Ahmed et al. (38) reported that optimum protease activity was 35°C for a *Bacillus cereus* strain isolated from soil. Abusham et al. (24) observed the highest growth and maximum protease production at 37 °C for *Bacillus subtilis* strain Rand. Similarly, Sharmin et al. (39) and Shumi et al. (40) reported that optimum temperature for cell growth and protease activity was 37°C for *Bacillus amovivorus* and for *Bacillus fastidious*, respectively. Higher incubation temperatures such as 47°C (41) and 55°C (42) were also reported as optimal temperatures for various *Bacillus subtilis* strains. The relation between energy metabolism and synthesis of enzymes has been reported to be controlled by temperature and oxygen uptake (43). Rahman et al. (35) reported that temperature effects the secretion of the extracellular enzyme via altering the cell membrane properties. With the gradual increase in temperature above 35°C, decrease in protease activity was reported by Asha and Palaniswamy (37). Contrary to this study, the optimum temperature for protease production was found to be 60°C for *Bacillus* sp. SMIA-2 (44) and 50°C for *Bacillus licheniformis* (45).

Although milk powder and casein are both milk based, it has been reported that different types of milk substrates could affect bacterial growth and proteolytic activity (46). Using milk powder instead of pure casein as a protein source in the growth media may slightly reduce protease activity, however, the low cost of milk powder could compensate for this loss exceedingly. Casein was also reported as the best protein source compared to other inorganic (ammonium acetate, sodium nitrate, ammonium chloride, potassium nitrate) and organic (soybean meal, yeast extract, peptone, beef extract, meat extract, skim milk powder) protein sources tested (37). They also used increased concentration of casein (up to 3.5%) in the production media and obtained protease activity ranging from 128.5 U/mL at 0.25% casein to 182.5 U/mL at 3.5% casein. In a study by Asha and Palaniswamy, among the protein sources tested, skim milk powder was the best option as protein source after casein (37). Sharma et al. (47) also observed higher protease production (49.77 U/ml) from bacterial strain AKS-4 when they used casein as a protein source. In another study, skim milk was found as the best substrate for the production of the extracellular protease (48). Different protein sources such as malt extract, peptone, yeast extract and corn steep liquor were also used for protease production by *Bacillus* species. While corn steep liquor yielded maximum protease activity for *Bacillus* sp. strain CR-179 (49), beef extract was the best substrate for protease production from *Bacillus cereus* strain 146 (50). It is known that protein source is an important component for enzyme production and different protein sources can be best for different bacterial strains for effective protease production (5, 51). Protein source and fermentation temperature proved to be important factors in protease activity which is also indicated by statistical analysis of our results. Specifically, casein and milk powder are significantly better inducers of protease secretion compared to gelatin and higher temperature also aids in higher protease activity in the final supernatant.

Another important parameter regarding protease productivity, especially from industrial perspective, is the fermentation time required for optimal protease activity. Kebabci et al. (52) reported maximum protease activity by novel *Bacillus cereus* at 18 h which occurred in the late stationary phase. Sepahy and Jabalamelikhan (49) observed that the maximum incubation time for protease activity was 24

h. In contrast, longer incubation periods such as 48 h (34) to 72 h (53) have been reported for maximum protease production. Although it is reported that synthesis of protease enzyme in *Bacillus* species occurred while the culture was metabolically active between exponential and the stationary growth phases (54, 55), according to Strauch et al. (56) and Gupta et al. (8) protease production starts at the beginning of stationary phase as soon as nutrient starvation occur. It can be inferred from these studies that optimum incubation time for protease activity changes depending on the *Bacillus* strain used.

Suitability of a strain for large scale production is a key characteristic for industrial applications (57). Production experiments in benchtop bioreactors is one of the first steps of assessment in this context. Our preliminary experiments showed that culture reaches a similar maximum OD in half the time compared to shake flask experiments. However, there was not a significant increase in protease activity, and it took longer fermentation time to achieve the maximum activity level. Effect of temperature, pH, media composition, aeration rate and agitation speed on bacterial growth and protease production have been extensively investigated in scale up studies (27, 58, 59). Both solid state and submerged fermentation methods (stirred tank and airlift bioreactors) were utilized resulting in a range of protease activity at various sets of growth conditions and feeding strategies (27, 60). Although fermentation in a bioreactor generally results in higher levels of proteolytic activity, elaborate optimization should be performed for each strain for maximum protease production. Therefore, to be able to utilize *Bacillus subtilis* strain NRRL B-3384 at industrial scale, further experiments are required.

Protease activity evaluation

According to experiments, increasing the incubation time with substrate resulted in decline of apparent protease activity. We attribute these results to substrate being exhausted in the first few minutes of the proteolysis reaction and the remaining time in long duration experiments being spent without proteolytic activity due to absence of substrate. Çoban (2016) (25) and Vaithanomsat et al. (26) used 20 min incubation time with substrate while Yang et al. (53) used 10 min incubation time.

Crude enzyme obtained from strain NRRL-B 3384 was able to retain 75% of its proteolytic activity at temperatures as high as 80°C. Similar to our results, *Bacillus* strains HR-08 and KR-8102 showed optimal activity at 65°C and 50°C, respectively (61) while strain DR8806 produce serine protease which was most active at only 45°C (62). Other studies also reported protease enzymes produced in various *Bacillus* strains to have different maximum activity temperatures such as 37°C (63), 50°C (64) and 55°C (65).

In terms of reaction pH/alkalinity, most proteases produced in *Bacillus* strains exhibit alkaline characteristics (66), and are also stable at neutral pH levels, however, some strains produce proteases that show drastic activity loss below pH 9 (64). Similar to this study, Farhadian et al. (62) found the enzyme produced by B. subtilis DR8806 was highly active across a pH range of 5 to 10.

Studies on the inhibitory and activating effects of various divalent cations are essential for better understanding the characteristics of an enzyme including nature of the active site and cofactor requirements (67). Different compounds had varying effects (both inhibitory and activating) on the enzyme (68).

Activating effects of Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} on protease activity has been reported previously (53, 69, 70). A different effect of a strong protease inhibition by Zn^{2+} was reported by Kalwasińska et al. (11) when using alkaline and halophilic protease from *Bacillus* luteus H11 and proteolytic activity was not much affected by by Ca^{2+} and Mg^{2+}. Decreasing activity in the presence of Mg^{2+}, Zn^{2+} and Fe^{2+} was observed by Çoban (25). Although EDTA has been mostly reported as an inhibitor for protease (11, 25, 53), some studies reported that EDTA did not show any significant changes on the protease activity (30, 44). Similar to our results, an activating effect by EDTA has been reported by Mothe et al. (71).

CONCLUSION

In the present study, two *Bacillus* subtilis spp. subtilis strains (NRRL B-3384 and NRRL B-3387) from ARS Culture collection (NRRL) repository were investigated for their protease production capabilities. Preliminary screening done on selective agar plates indicated that the strain NRRL B-3384 was the better candidate for protease production with the highest clear zone to colony size ratio on different protein sources. Enzymatic activity was retained at elevated temperatures and was maximal at high pH suggesting that one or more secreted proteases are of alkaline character. Among various protein sources, casein showed the highest induction of protease activity, although milk powder proved to be a reasonable alternative for industrial manufacturing due to its lower cost. The thermal stability and presence of enzymatic activity across a broad range of pH and divalent cations suggest that protease(s) from the NRRL B-3384 strain could be useful for various industries such as textile, detergent, and wastewater treatment. Preliminary experiments with laboratory-scale bioreactors suggest that, with additional optimization, NRRL B-3384 may be suitable for

large-scale production of proteolytic enzymes.

ACKNOWLEDGEMENTS

Author would like to thank Dr. Hasan Bugra COBAN from Dokuz Eylül University, for his helpful discussions for improving this article and Dr. Irem DENIZ CAN from Manisa Celal Bayar University for her support in laboratory facilities and equipment. Author would like to thank Prof. Dr. Theodore G. CLARK from Cornell University for his valuable comments and editing the final version of the manuscript.

CONFLICT OF INTEREST

The author declares no conflict of interest.

References

1. Jegannathan KR, Nielsen PH. Environmental assessment of enzyme use in industrial production–a literature review. Journal of cleaner production. 2013;42:228-40.
2. Singh S, Bajaj BK. Bioprocess optimization for production of thermoalkali-stable protease from Bacillus subtilis K-1 under solid-state fermentation. Preparative Biochemistry and Biotechnology. 2016;46(7):717-24.
3. Razzaq A, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A, et al. Microbial proteases applications. Frontiers in bioengineering and biotechnology. 2019;7:110.
4. Singhal P, Nigam V, Vidyarthi A. Studies on production, characterization and applications of microbial alkaline proteases. International Journal of Advanced Biotechnology and Research. 2012;3(3):653-69.
5. Sharma KM, Kumar R, Panwar S, Kumar A. Microbial alkaline proteases: Optimization of production parameters and their properties. Journal of Genetic Engineering and Biotechnology. 2017;15(1):115-26.
6. Pihlanto A. Antioxidative peptides derived from milk proteins. International dairy journal. 2006;16(11):1306-14.
7. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiology and molecular biology reviews. 1998;62(3):597-635.
8. Gupta R, Beg Q, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Applied microbiology and biotechnology. 2002;59(1):15-32.
9. Contesini FJ, Melo RRd, Sato HH. An overview of Bacillus proteases: from production to application. Critical reviews in biotechnology. 2018;38(3):321-34.
10. Helal M, Amer H, Abdelwahed N, Ghobashy M. Physiological and microbiological studies on production of alkaline protease from locally isolated Bacillus subtilis. Aust J Basic Appl Sci. 2012;6:193-203.
11. Kalwasińska A, Jankiewicz U, Felföldi T, Burkowska-But A, Swiontek Brzezinska M. Alkaline and halophilic protease production by Bacillus luteus H11 and its potential industrial applications. Food technology and biotechnology. 2018;56(4):553-61.
12. Joo HS, Chang CS. Oxidant and SDS-stable alkaline protease from a halo-tolerant Bacillus clausii I-52: enhanced production and simple purification. Journal of applied microbiology. 2005;98(2):491-7.
13. Ibrahim KS, Muniyandi J, Pandian SK. Purification and characterization of manganese-dependent alkaline serine protease from Bacillus pumilus TMS55. Journal of microbiology and biotechnology. 2011;21(1):20-7.
14. Baweja M, Tiwari R, Singh PK, Nain L, Shukla P. An alkaline protease from Bacillus pumilus MP 27: functional analysis of its binding model toward its applications as detergent additive. Frontiers in microbiology. 2016;7:1195.
15. Haddar A, Bougatef A, Agrebi R, Sellami-Kamoun A, Nasri M. A novel surfactant-stable alkaline serine-protease from a newly isolated Bacillus mojavensis A21. Purification and characterization. Process Biochemistry. 2009;44(1):29-35.
16. Saggu SK, Mishra PC. Characterization of thermostable alkaline proteases from Bacillus infantis SKS1 isolated from garden soil. PloS one. 2017;12(11):e0188724.
17. Anbu P. Characterization of solvent stable extracellular protease from Bacillus koreensis (BK-P21A). International Journal of Biological Macromolecules. 2013;56:162-8.
18. Singh J, Vohra R, Sahoo DK. Enhanced production of alkaline proteases by Bacillus sphaericus using fed-batch culture. Process Biochemistry. 2004;39(9):1093-101.
19. Moon SH, Parulekar SJ. A parametric study ot protease production in batch and fed-batch cultures of Bacillus firmus. Biotechnology and bioengineering. 1991;37(5):467-83.
20. Ferreira L, Ramos M, Dordick J, Gil M. Influence of different silica derivatives in the immobilization and stabilization of a Bacillus licheniformis protease (Subtilisin Carlsberg). Journal of Molecular Catalysis B: Enzymatic. 2003;21(4-6):189-99.
21. Yilmaz B, Baltaci MO, Sisecioglu M, Adiguzel A. Thermotolerant alkaline protease enzyme from Bacillus licheniformis A10: purification, characterization, effects of surfactants and organic solvents. Journal of enzyme inhibition and medicinal chemistry. 2016;31(6):1241-7.
22. Pohl S, Harwood CR. Heterologous protein secretion by Bacillus species: from the cradle to the grave. Advances in applied microbiology. 2010;73:1-25.
23. Cui W, Han L, Suo F, Liu Z, Zhou L, Zhou Z. Exploitation of Bacillus subtilis as a robust workhorse for production of heterologous proteins and beyond. World Journal of Microbiology and Biotechnology. 2018;34(10):1-19.
24. Abusham RA, Rahman RNZR, Salleh AB, Basri M. Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant Bacillus subtilis strain Rand. Microbial Cell Factories. 2009;8(1):1-9.
25. ÇOBAN HB. Axinella damicornis süngerinden izole edilen sucul bakterilerin proteaz üretkenliklerinin araştırılması ve üretilen proteaz enziminin kısmi karakterizasyonu. Mediterranean Agricultural Sciences.33(2):223-9.
26. Vaithanomsat P, Malapant T, Apiwattanapiwat W. Silk degumming solution as substrate for microbial protease production. Agriculture and Natural Resources. 2008;42(3):543-51.
27. Prasad R, Abraham TK, Nair AJ. Scale up of production in a bioreactor of a halotolerant protease from moderately halophilic Bacillus sp. isolated from soil. Brazilian Archives of Biology and Technology. 2014;57(3):448-55.
28. VijayAnand S, Hemapriya J, Selvin J, Kiran S. Production and optimization of haloalkaliphilic protease by an extremophile-Halobacterium sp. Js1, isolated from thalassohaline environment. Global J Biotechnol Biochem. 2010;5(1):44-9.
29. Ali N, Ullah N, Qasim M, Rahman H, Khan SN, Sadiq A, et al.

Molecular characterization and growth optimization of halo-tolerant protease producing Bacillus subtilis Strain BLK-1.5 isolated from salt mines of Karak, Pakistan. Extremophiles. 2016;20(4):395-402.
30. Adinarayana K, Ellaiah P, Prasad DS. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated Bacillus subtilis PE-11. Aaps Pharmscitech. 2003;4(4):440-8.
31. Nihalani D, Satyanarayana T. Isolation and characterization of extracellular alkaline enzyme producing bacteria. Indian journal of microbiology New Delhi. 1992;32(4):443-9.
32. Gessesse A, Gashe BA. Production of alkaline protease by an alkaliphilic bacteria isolated from an alkaline soda lake. Biotechnology Letters. 1997;19(5):479-81.
33. Pant G, Prakash A, Pavani J, Bera S, Deviram G, Kumar A, et al. Production, optimization and partial purification of protease from Bacillus subtilis. Journal of Taibah University for Science. 2015;9(1):50-5.
34. Khan I, Gupta P, Vakhlu J. Thermo-alkaliphilic halotolerant detergent compatible protease (s) of Bacillus tequilensis MTCC 9585. African Journal of Microbiology Research. 2011;5(23):3968-75.
35. Abd Rahman RNZ, Geok LP, Basri M, Salleh AB. Physical factors affecting the production of organic solvent-tolerant protease by Pseudomonas aeruginosa strain K. Bioresource technology. 2005;96(4):429-36.
36. Gouda MK. Optimization and purification of alkaline proteases produced by marine Bacillus sp. MIG newly isolated from Eastern Harbour of Alexandria. Polish Journal of microbiology. 2006;55(2):119.
37. Asha B, Palaniswamy M. Optimization of alkaline protease production by Bacillus cereus FT 1 isolated from soil. J App Pharm Sci. 2018;8(02):119-27.
38. Ahmed M, Rehman R, Siddique A, Hasan F, Ali N, Hameed A. Production, purification and characterization of detergent-stable, halotolerant alkaline protease for eco-friendly application in detergents' industry. Int J Biosci. 2016;8(2),:47-65.
39. Sharmin S, Hossain MT, Anwar M. Isolation and characterization of a protease producing bacteria Bacillus amovivorus and optimization of some factors of culture conditions for protease production. J Biol Sci. 2005;5(3):358-62.
40. Shumi W, Hossain MT, Anwar M. Proteolytic activity of a bacterial isolate Bacillus fastidiosus den Dooren de Jong. J biol Sci. 2004;4(3):370-4.
41. Chantawannakul P, Oncharoen A, Klanbut K, Chukeatirote E, Lumyong S. Characterization of proteases of Bacillus subtilis strain 38 isolated from traditionally fermented soybean in Northern Thailand. Science Asia. 2002;28(4):241-5.
42. Rehman R, Ahmed M, Siddique A, Hasan F, Hameed A, Jamal A. Catalytic role of thermostable metalloproteases from Bacillus subtilis KT004404 as dehairing and destaining agent. Applied biochemistry and biotechnology. 2017;181(1):434-50.
43. Frankena J, Koningstein GM, van Verseveld HW, Stouthamer AH. Effect of different limitations in chemostat cultures on growth and production of exocellular protease by Bacillus licheniformis. Applied microbiology and biotechnology. 1986;24(2):106-12.
44. Nascimento WCAd, Martins MLL. Production and properties of an extracellular protease from thermophilic Bacillus sp. Brazilian journal of microbiology. 2004;35:91-6.
45. Abdulrahman A, Yasser M. Production and some properties of protease produced by Bacillus licheniformis isolated from Tihamet Aseer, Saudi Arabia. Pakistan Journal of Biological Sciences (Pakistan). 2004.
46. Colantuono A, D'Incecco P, Fortina MG, Rosi V, Ricci G, Pellegrino L. Milk substrates influence proteolytic activity of Pseudomonas fluorescens strains. Food Control. 2020;111:107063.
47. Sharma AK, Sharma V, Saxena J, Yadav B, Alam A, Prakash A. Optimization of protease production from bacteria isolated from soil. Appl Res J. 2015;1(7):388-94.
48. Uyar F, Porsuk I, Kizil G, Yilmaz EI. Optimal conditions for production of extracellular protease from newly isolated Bacillus cereus strain CA15. EurAsian Journal of BioSciences. 2011;5.
49. Akhavan Sepahy A, Jabalameli L. Effect of culture conditions on the production of an extracellular protease by Bacillus sp. isolated from soil sample of Lavizan Jungle Park. Enzyme research. 2011;2011.
50. Shafee N, Aris SN, Rahman R, Basri M, Salleh AB. Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium Bacillus cereus strain 146. J Appl Sci Res. 2005;1(1):1-8.
51. Mehta V, Thumar J, Singh S. Production of alkaline protease from an alkaliphilic actinomycete. Bioresource technology. 2006;97(14):1650-4.
52. Kebabci Ö, Cihangir N. Full Length Research Paper Isolation of protease producing novel Bacillus cereus and detection of optimal conditions. African Journal of Biotechnology. 2011;10(7):1160-4.
53. Yang J-K, Shih L, Tzeng Y-M, Wang S-L. Production and purification of protease from a Bacillus subtilis that can deproteinize crustacean wastes☆. Enzyme and microbial technology. 2000;26(5-6):406-13.
54. Priest FG. Extracellular enzyme synthesis in the genus Bacillus. Bacteriological reviews. 1977;41(3):711-53.
55. Prestidge L, Gage V, Spizizen J. Protease activities during the course of sporulation in Bacillus subtilis. Journal of Bacteriology. 1971;107(3):815-23.
56. Strauch MA, Hoch JA. Transition-state regulators: sentinels of Bacillus subtilis post-exponential gene expression. Molecular microbiology. 1993;7(3):337-42.
57. Wehrs M, Tanjore D, Eng T, Lievense J, Pray TR, Mukhopadhyay A. Engineering robust production microbes for large-scale cultivation. Trends in microbiology. 2019;27(6):524-37.
58. Yuguo Z, Zhao W, Xiaolong C, Chunhua Z. Production of Extracellular Protease from Crude Substrates with Dregs in an External-Loop Airlift Bioreactor with Lower Ratio of Height to Diameter. Biotechnology progress. 2001;17(2):273-7.
59. Potumarthi R, Ch S, Jetty A. Alkaline protease production by submerged fermentation in stirred tank reactor using Bacillus licheniformis NCIM-2042: effect of aeration and agitation regimes. Biochemical Engineering Journal. 2007;34(2):185-92.
60. Beg QK, Sahai V, Gupta R. Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor. Process Biochemistry. 2003;39(2):203-9.
61. Moradian F, Khajeh K, Naderi-Manesh H, Ahmadvand R, Sajedi RH, Sadeghizadeh M. Thiol-dependent serine alkaline proteases from Bacillus sp. HR-08 and KR-8102. Applied biochemistry and biotechnology. 2006;134(1):77-87.
62. Farhadian S, Asoodeh A, Lagzian M. Purification, biochemical characterization and structural modeling of a potential htrA-like serine protease from Bacillus subtilis DR8806. Journal of Molecular Catalysis B: Enzymatic. 2015;115:51-8.
63. Cha M, Park JR, Yoon KY. Purification and characterization of an alkaline serine protease producing angiotensin I-converting enzyme inhibitory peptide from Bacillus sp. SS103. Journal of medicinal food. 2005;8(4):462-8.
64. Giri SS, Sukumaran V, Sen SS, Oviya M, Banu BN, Jena PK. Purification and partial characterization of a detergent and

oxidizing agent stable alkaline protease from a newly isolated Bacillus subtilis VSG-4 of tropical soil. The Journal of Microbiology. 2011;49(3):455-61.

65. Huang Q, Peng Y, Li X, Wang H, Zhang Y. Purification and characterization of an extracellular alkaline serine protease with dehairing function from Bacillus pumilus. Current microbiology. 2003;46(3):0169-73.
66. Mane R, Bapat M. A study of extracellular alkaline protease from Bacillus subtilis NCIM 2713. 2001.
67. Sigman DS, Mooser G. Chemical studies of enzyme active sites. Annual Review of Biochemistry. 1975;44(1):889-931.
68. de Cassia Pereira J, Giese EC, de Souza Moretti MM, dos Santos Gomes AC, Perrone OM, Boscolo M, et al. Effect of metal ions, chemical agents and organic compounds on lignocellulolytic enzymes activities. Enzyme inhibitors and activators. 2017;29:139-64.
69. Yossan S, Reungsang A, Yasuda M. Purification and characterization of alkaline protease from Bacillus megaterium isolated from Thai fish sauce fermentation process. Science Asia. 2006;32(4):377-83.
70. Deng A, Wu J, Zhang Y, Zhang G, Wen T. Purification and characterization of a surfactant-stable high-alkaline protease from Bacillus sp. B001. Bioresource technology. 2010;101(18):7100-6.
71. Mothe T, Sultanpuram VR. Production, purification and characterization of a thermotolerant alkaline serine protease from a novel species Bacilluscaseinilyticus. 3 Biotech. 2016;6(1):53.