RESEARCH ARTICLE

Evaluation of extracellular alkaline proteases from *Bacillus* for environmentally friendly detergent additives

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Abstract: Growing demand for environmentally friendly and sustainable enzyme solutions drives markets for alkaline proteases across various industries. Alkaline proteases produced by different *Bacillus* strains exhibit unique properties and versatility. Production of alkaline proteases from *Bacillus amyloliquefaciens* TBRC 2902, *B. siamensis* TBRC 1180, *B. subtilis* TBRC 6663, and *B. velezensis* TBRC 7773 was optimized by adjusting production media, considering carbon and nitrogen sources to enhance extracellular enzyme activities. Optimized extracellular protease activity was achieved using tapioca starch for TBRC 2902 and TBRC 1180, and soluble starch for TBRC 7773, while both carbon sources were optimal for TBRC 6663. Skim milk was an equally effective nitrogen source for TBRC 2902, TBRC 1180, and TBRC 7773, whereas soytone was as effective as yeast extract for TBRC 6663. Inorganic nitrogen sources, such as diammonium hydrogen phosphate for TBRC 2902 and potassium nitrate for TBRC 1180, enhanced alkaline protease activity by 10-20%. The protease from *B. siamensis* TBRC 1180 exhibited optimal pH and temperature values of 9.0 and 60°C, respectively, while the others had optimal values of 8.0 and 50°C. All enzymes tolerated non-ionic surfactants, retaining over 40% activity after 24 hours exposure to Triton X-100, Tween-20, and Tween-80, indicating their potential as detergent additives.
Keywords: *Bacillus amyloliquefaciens*, *Bacillus siamensis*, *Bacillus subtilis*, *Bacillus velezensis*, alkaline proteases, surfactants

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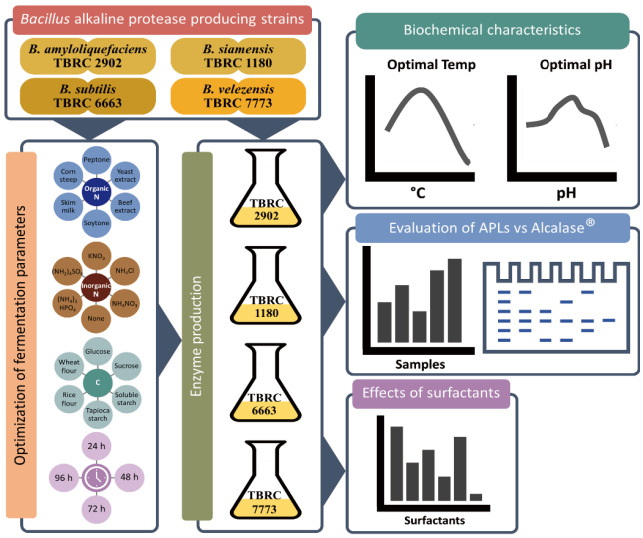
Received: May 23, 2024; **Accepted:** June 20, 2024; **Published Online:** July 2, 2024
Citation: Prabmark, K., Saeng-kla, K., Boonyapakron, K., Aiewviriyasakul, K., Sritusnee, W., Bunterngsook, B., Poncheewin, W., Kanokratana, P., Champreda, V., Laothanachareon, T., 2024. Evaluation of extracellular alkaline proteases from *Bacillus* for environmentally friendly detergent additives. *Applied Environmental Biotechnology*, 9(1): 26-37. http://doi.org/10.26789/AEB.2024.01.004

Highlights

- Alkaline proteases characterized from cultures of *Bacillus amyloliquefaciens*, *B. siamensis*, *B. subtilis*, and *B. velezensis*.
- Tapioca and soluble starch were optimal carbon sources for protease production.
- Yeast extract was the optimal organic nitrogen source for production of all proteases.
- Skim milk and soytone identified as alternative optimal organic nitrogen sources for protease production.
- Thermo-alkaliphilic optimal activities demonstrated for crude enzymes.
- Tolerance to surfactants demonstrated for crude enzymes.



Graphical Abstract

1 Introduction

Proteases (EC: 3.4.21-24 and 99) hydrolyze peptide bonds within complex protein compounds into amino acids and peptides and are widely used in various industries, accounting for approximately 60% of the global industrial enzyme usage (Masi et al., 2021; Pham et al., 2022; Ullah et al., 2022). Proteases are categorized as acidic, alkaline, and neutral proteases based on their optimal effectiveness within specific pH ranges (Rao et al., 1998). Alkaline proteases are used in leather, textile, food, and pharmaceutical industries (Gupta et al., 2002; Yilmaz et al., 2016). Moreover, alkaline proteases can be used in detergent formulations to enhance the washing performance (Grbavčić et al., 2011). Alkaline proteases in detergent formulations effectively eliminate protein-based stains, such as blood, food, and grass stains (Wang et al., 2007; Smulders and Sung, 2011; Asha and Palaniswamy, 2018).

Alkaline proteases can be obtained from various sources, including insects (silkworms and termites), plants (papaya and pineapple), fungi (*Aspergillus*, *Penicillium*, and *Rhizopus*), and bacteria (*Bacillus*) (Gupta et al., 2002; Sharma et al., 2019; Kannan et al., 2019; Errasti et al., 2020; Pawar et al., 2023). Among these sources, *Bacillus* spp. are recognized as highly active and efficient producers of extracellular alkaline proteases. Numerous *Bacillus* alkaline proteases have been isolated and characterized for their biochemical properties, including *B. clausii* (Joo et al., 2005), *B. koreensis* (Anbu, 2013), *B. laterosporus* (Arulmani et al., 2007), *B. licheniformis* (Sareen and Mishra, 2008; Sarker et al., 2013; Emran et al., 2020), *B. mojavensis* (Haddar et al., 2009a), *B. pumilus* (Jaouadi et al., 2008), and *B. subtilis* (Mashayekhi, 2012; Sathishkumar et al., 2015; Farhadian et al., 2015). Despite several reports on alkaline proteases from *Bacillus*, new strains with superior fermentation characteristics and enzyme production for industrial applicability are desirable (Puri et al., 2002; Bhunia et al., 2012).

In this study, strains of four *Bacillus* species, namely, *B. amyloliquefaciens*, *B. siamensis*, *B. subtilis*, and *B. velezensis*, were selected for the study of their alkaline proteases. Different media and cultivation conditions were tested for optimization of alkaline protease production, and the biochemical properties of the enzymes were characterized. Enzyme activities in the presence of ionic and non-ionic surfactants were studied to test their applicability as additives in detergent formulations.

2 Materials and Methods

2.1 Strains and media

B. *amyloliquefaciens* TBRC 2902, *B. siamensis* TBRC 1180, *B. subtilis* TBRC 6663, and *B. velezensis* TBRC 7773 were obtained from the Thailand Bioresource Research Center (www.tbrcnetwork.org). Bacterial cultures were maintained on Luria-Bertani (LB) agar containing 10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar at 30°C. The inoculum was prepared by transferring a single colony from the plate culture to 5 mL of LB medium in a sterilized 50 mL centrifuge tube. The cultures were incubated at 30°C for 24 hours with shaking at 200 rpm. Chemicals were procured from major chemical suppliers (Sigma, Fluka, and Merck). Culture medium components were obtained from BD Difco. Tapioca starch, rice flour, and wheat flour were procured from a local market.

2.2 Optimization of culture conditions for protease production

To enhance alkaline protease activities, each strain was individually optimized for its specific media compositions. The optimization process involved sequential adjustments, beginning with organic nitrogen sources, followed by inorganic nitrogen sources, carbon sources, and incubation time. The effect of nitrogen sources was examined by adding 10 g/L of various organic nitrogen sources, including peptone, yeast extract, beef extract, soy tone, skim milk, and corn steep liquor into the basal medium (1 g/L K_2HPO_4, 0.2 g/L $MgSO_4$, and 10 g/L glucose). Similarly, different inorganic nitrogen sources such as KNO_3, NH_4Cl, NH_4NO_3, $(NH_4)_2HPO_4$, and $(NH_2)_4SO_4$ were added to the basal medium with the optimal organic nitrogen source of each strain. The impact of the carbon source was investigated by adding 10 g/L of various carbon sources (glucose, sucrose, soluble starch, tapioca starch, rice flour, and wheat flour) in conjunction with the optimal organic and inorganic nitrogen sources of each strain.

A 2% (v/v) overnight culture, cultivated in LB medium was inoculated into each cultivation medium mixture. Subsequently, 1.5 mL of the inoculated medium was added to wells of a 48-well-flower plate (m2p labs, Germany) and incubated in a m2p-labs BioLector® microbioreactor (m2p labs) at 30°C for 48 hours with shaking at 1,000 rpm. Culture samples were centrifuged at 12,000×g for 5 min and the supernatants were transferred to 1.5 mL microcentrifuge tubes. The effects of nitrogen and carbon sources on protease production were measured by colorimetric protease activity assay using casein (Sigma Aldrich, MA, USA) as a substrate in 50 mM Tris-HCl buffer solution at pH 9.0.

The effect of incubation time on protease activity was studied by inoculating the 2.0% (v/v) overnight cultures from LB medium into a 50 mL centrifuge tube containing optimal medium. Protease enzyme production was performed at 30°C in a rotary shaker at 250 rpm. Culture samples were taken after 0, 24, 48, 72, and 96 hours, and the supernatants were collected by centrifugation at 12,000×g for 5 min. The protease activity was analyzed by colorimetric protease activity assay using casein as a substrate in 50 mM Tris-HCl buffer solution at pH 9.0. The optimal parameters, including nitrogen and carbon sources, as well as the incubation time

for each strain were applied to prepare the crude protease enzymes for further studies.

2.3 Protease activity assay

Protease activity was determined by incubating 100 μL of the culture supernatant crude enzyme, prepared using the optimal medium compositions, with 500 μL of 0.65% (w/v) casein (Sigma Aldrich) in 50 mM Tris-HCl (pH 9.0). The incubation took place at 40°C for 10 min, following a standard method (Folin and Ciocalteu, 1927). The reactions were stopped by adding 500 μL of 110 mM trichloroacetic acid (TCA) (Sigma Aldrich) and centrifugation at 12,000×g for 5 min. After adding 500 μL of 500 mM sodium carbonate (Na_2CO_3) (Sigma Aldrich) to 200 μL of the supernatant, 100 μL of 20% (v/v) Folin & Ciocalteu's phenol reagent (Sigma Aldrich) was added to the mixture and incubated at 37°C for 30 min. The liberated tyrosine was measured at 660 nm. A control was run in the same manner, except the crude enzyme was added after the addition of 110 mM TCA. The calibration curve was produced using tyrosine (Sigma Aldrich) as a standard from 0 to 20 μg.

One unit of protease activity (U/mL) was defined as the amount of enzyme that liberated 1.0 μg of tyrosine per minute under the test conditions. Activity assays were conducted in triplicate; results shown in figures are mean values with associated standard deviations. The specific activities of the proteases from all tested strains on casein were determined using 50 mM Tris-HCl buffer at pH 9.0 and 40°C. Group-wise mean differences were analysed by One-way ANOVA analysis of variance using Tukey's Honest Significant Difference (HSD) test. *P*-values less than 0.05 were considered statistically significant.

Alcalase® commercial alkaline protease (Novozymes, Denmark) was used as a benchmark enzyme for comparison of the protease activities produced by the four *Bacillus* species. The total protein concentrations of the crude enzymes and Alcalase® were determined by Bradford assay (Bio-Rad, CA, US) using bovine serum albumin (BSA) as a standard (Bio Basic Inc., Canada). The absorbance of the reaction mixtures was measured after 5 min of incubation at room temperature at a wavelength of 595 nm using a BioTek Synergy H1 Microplate Reader (Agilent Technologies, CA, US). The molecular weights of the major protein species present in the supernatants were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized by staining with InstantBlue® Coomassie Protein Stain (Abcam, UK).

2.4 Effects of pH and temperature

The crude enzymes from each strain were individually prepared using the optimal medium compositions. Enzyme activities were tested over the pH range 6-12 controlled using buffers at a concentration of 50 mM, including sodium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 7-9), and glycine-NaOH buffer (pH 9-12). The protease enzyme activity was measured as described above. The enzyme activities at different pH were normalized to the maximum activity measured for each enzyme. Similarly, the temperature dependency of the enzymes was investigated by incubating the reaction mixture at temperatures ranging from 25 to 80°C in the pH-optimal buffer that provided the greatest activity for each enzyme.

2.5 Tolerance to surfactants for detergent application

The effect of different surfactants on the enzyme stability was tested by incubating culture supernatant crude enzyme samples with 5% Tween-20, 5% Tween-80, 5% Triton X-100, 1% CTAB, and 0.5% SDS at 30°C for 24 hours. The residual enzyme activity was determined using the colorimetric assay with casein as the substrate at 40°C in 50 mM Tris-HCl buffer solution at pH 9.0. The relative activity (%) was calculated by comparing the enzyme activities remaining in the presence of each surfactant to the activity of the enzyme incubated with sterile water at the starting point (T_0), which was used as a control.

3 Results

3.1 Optimization of culture conditions for protease production

Four strains of *Bacillus* spp. from the TBRC culture collection, previously identified as potential producers of extracellular alkaline protease, were selected for this study. The production of alkaline protease from these strains was tested under culture conditions with different nitrogen and carbon sources. Among the six organic nitrogen sources tested, cultures with yeast extract produced the highest protease activity among all strains, reaching levels exceeding 78 U/mL, which was significantly greater than that produced in cultures with other organic nitrogen sources (Figure 1A-D), except for cultures of *B. subtilis* TBRC 6663 with soytone (80.06±3.30 U/mL) and TBRC 7773 with skim milk (78.33±7.68 U/mL), respectively, which produced protease activities not significantly different from cultures of the same strains containing yeast extract.

The addition of inorganic nitrogen sources had a slightly inducing effect on the protease activity of certain strains. The inorganic nitrogen sources were found to enhance the protease activity of TBRC 2902 by 10 - 20%. Notably, the supplementation of $(NH_4)_2HPO_4$ resulted in the highest increase, reaching 20.84%, in protease activity for TBRC 2902, compared with the control condition without inorganic nitrogen source (Figure 1E). Likewise, the supplementation of KNO_3, NH_4Cl, NH_4NO_3 and $(NH_2)_4SO_4$ could induce the protease activity of TBRC 1180 by around 4% to 11%.

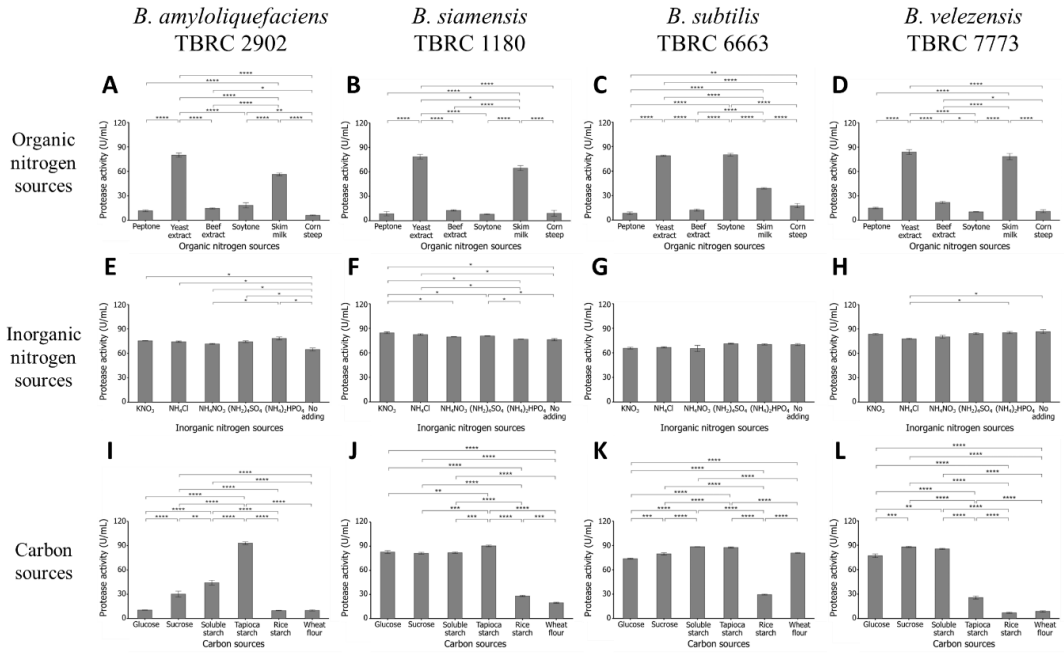


Figure 1. Optimization of culture conditions for alkaline protease activity. The crude enzymes were produced from *B. amyloliquefaciens* TBRC 2902 (A, E, and I), *B. siamensis* TBRC 1180 (B, F, and J), *B. subtilis* TBRC 6663 (C, G, and K), and *B. velezensis* TBRC 7773 (D, H, and L). The studied parameters included organic nitrogen sources (A-D), inorganic nitrogen sources (E-H), and carbon sources (I-L). Asterisks represent statistically significant difference (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, and **** p-value < 0.0001).

Among these, KNO_3 was identified as the most effective inorganic nitrogen source for this strain. This inorganic nitrogen source increased the activity by up to 11.15% compared to the culture without the inorganic nitrogen sources (Figure 1F). On the contrary, the addition of the tested inorganic nitrogen source was unnecessary for protease production for TBRC 6663 and TBRC 7773 (Figure 1G-H).

In terms of carbon sources, addition of glucose and sucrose strongly increased protease activity of TBRC 1180, TBRC 6663, and TBRC 7773, with the levels exceeding 70 U/mL. However, these carbon sources could not enhance the protease activity of strain TBRC 2902. Cultures with tapioca starch show a significantly greater production compared with all other carbon sources for TBRC 2902 and TBRC 1180 and can be considered the optimal carbon source for these strains (Figure 1I-J). In contrast, protease activity was greatest in the culture with soluble starch for TBRC 6663, which was not significantly different from that with tapioca starch (Figure 1k). For TBRC 7773, protease activity was greatest with sucrose, which was not significantly different from that with soluble starch (Figure 1L).

The incubation time was investigated in the range of 24 to 96 hours. The highest protease activity for TBRC 1180 and TBRC 6663 was observed after 24 hours, whereas peak activity was not observed until 48 hours for TBRC 2902 and TBRC 7773 (Figure 2). The protease activities of TBRC 2902 and TBRC 7773 dramatically decreased after 96 hours. In contrast, the protease activities of TBRC 1180 and TBRC 6663 were maintained at high levels (97% and 92%, respectively).

3.2 Characterization of crude protease from each *Bacillus* strain

The optimal pH and temperature for activity of the four extracellular proteases were determined. The protease from strain TBRC 2902 exhibited activity over a broad pH range from pH 7.0 to 12.0, exceeding 94.7% of maximum activity at all pH values tested (Figure 3A). The optimal pH of the TBRC 2902 crude enzyme was pH 8.0 in Tris-HCl buffer. It exhibited a relative activity at 79.5% in sodium phosphate buffer at pH 6.0 and its activity remained high at 95.9% in glycine-NaOH at pH 12.0. TBRC 6663 and TBRC 7773 crude enzymes showed the optimal pH at 8.0 in Tris-HCl buffer, but their working pH ranges were 7.0-11.0 and 7.0-9.0, respectively, which were narrower than the range of the TBRC 2902 enzyme (Figure 3C-D). The protease from *B. velezensis* TBRC 7773 was less tolerant of extreme alkalinity, as its activity dropped below 75.0% when the pH exceeded 9.0. In contrast, the TBRC 1180 protease was optimal in Tris-HCl and glycine-NaOH pH 9.0, and the working pH range (with the residual activities above 90.0%) was 7.0 to 11.0 (Figure 3B).

The protease activities of each strain showed similar temperature profiles (Figure 4). The optimal temperature was 50°C for all enzymes, except for the TBRC 1180 enzyme, which showed an optimal temperature of 60°C. However, all enzymes shared a common working temperature range of

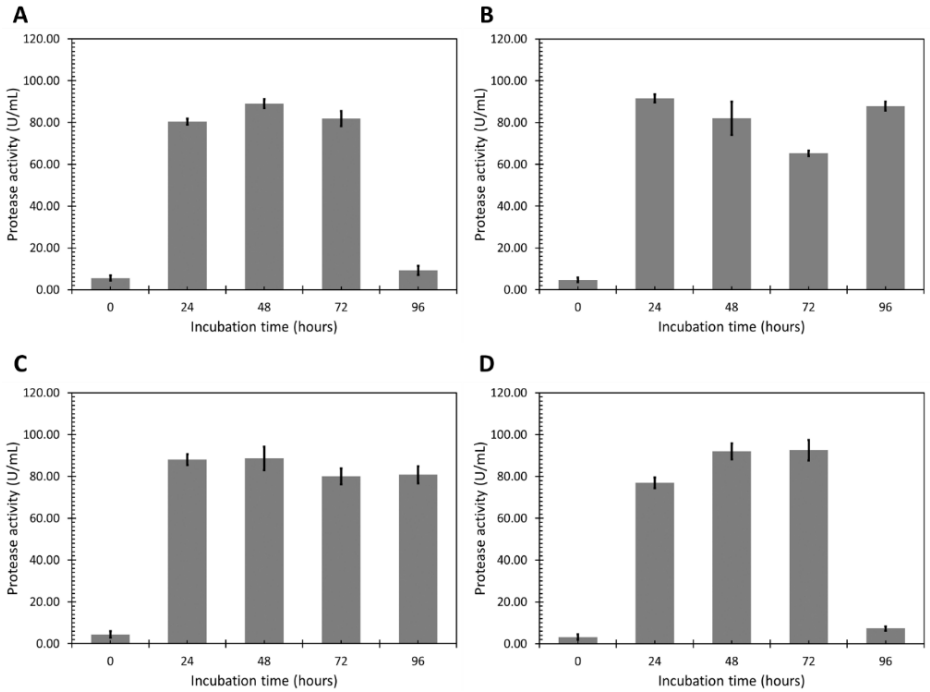


Figure 2. Optimization of incubation time for the highest alkaline protease activity. The crude enzymes were produced from *B. amyloliquefaciens* TBRC 2902 (A), *B. siamensis* TBRC 1180 (B), *B. subtilis* TBRC 6663 (C), and *B. velezensis* TBRC 7773 (D). The samples were collected at 24, 48, 72, and 96 hours. The assay was performed in 50 mM Tris-HCl pH 9.0 at 30°C.

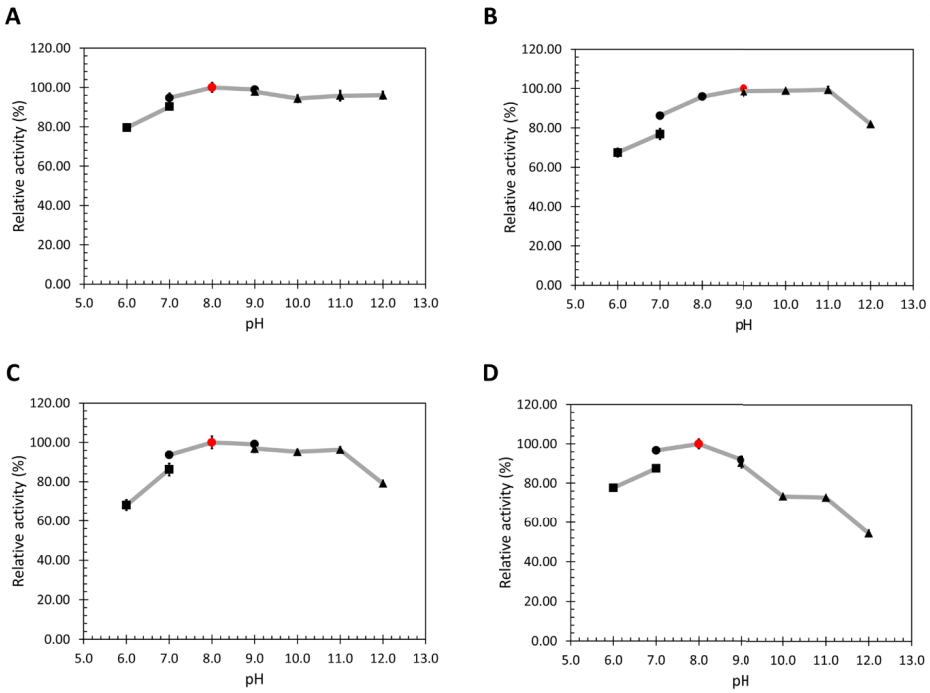


Figure 3. pH profiles of protease activity in crude enzymes produced from *B. amyloliquefaciens* TBRC 2902 (A), *B. siamensis* TBRC 1180 (B), *B. subtilis* TBRC 6663 (C), and *B. velezensis* TBRC 7773 (D), using casein as the substrate. Squares represent assays performed with 50 mM sodium phosphate buffer, circles represent assays with Tris-HCl buffer, and triangles represent assays with glycine-NaOH buffer. The assay was performed at 40°C. Red indicates 100% relative activity of each strain.

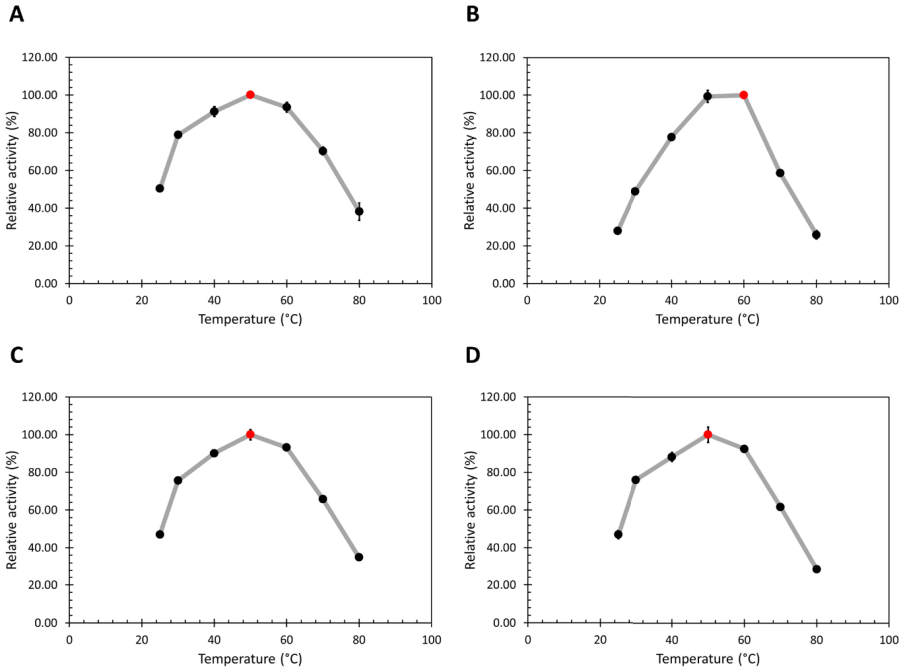


Figure 4. Temperature profiles of protease activity in crude enzymes produced from *B. amyloliquefaciens* TBRC 2902 (A), *B. siamensis* TBRC 1180 (B), *B. subtilis* TBRC 6663 (C), and *B. velezensis* TBRC 7773 (D), using casein as the substrate. The assay was performed in 50 mM Tris-HCl pH 9.0. Red indicates 100% relative activity of each strain.

40 to 60°C, with residual activities exceeding 80%. At the ambient temperature of 25°C, the protease activities of all strains declined below 50%, while at the higher temperature of 80°C, the activities dropped to less than 40%.

The specific activities of the proteases from all tested strains on casein were determined using 50 mM Tris-HCl buffer at pH 9.0 and 40°C and compared with Alcalase, a well-known commercial enzyme produced by Novozymes. Alcalase, produced by *B. licheniformis*, exhibits a working pH range of 6.5-10.0 and a working temperature range of 60-75°C. The specific activities of crude enzymes from all the tested strains were significantly lower than that of Alcalase (15.22±0.31 U/mg), and significant differences were observed in all pairwise comparisons of crude enzymes. Notably, the crude enzyme from *B. velezensis* TBRC 7773 exhibited the highest specific protease activity of 12.16±0.42 U/mg, which was approximately 20% lower than that of Alcalase (Figure 5A). The crude enzymes exhibited a complex protein profile with multiple bands observed in SDS-PAGE migrating 25 to 70 kDa, in contrast to the single band migrating at 32 kDa for Alcalase (Figure 5B).

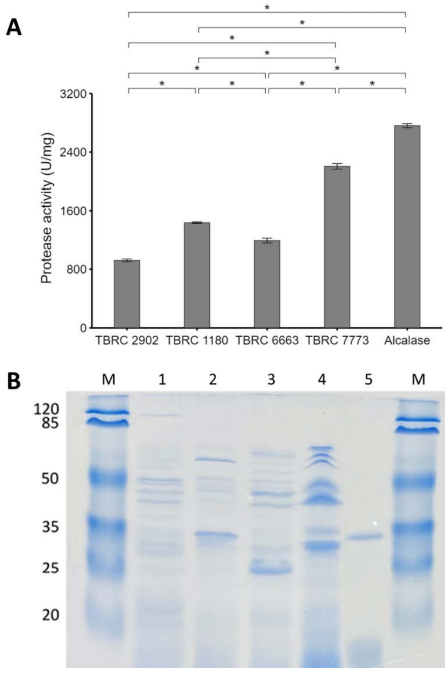


Figure 5. Comparison of crude proteases with Alcalase. Specific protease activities of crude enzyme produced by *B. amyloliquefaciens* TBRC 2902, *B. siamensis* TBRC 1180, *B. subtilis* TBRC 6663, and *B. velezensis* TBRC 7773 were compared against each other and Alcalase, a commercial purified enzyme (A). Asterisks denote statistically significant differences (p-value < 0.05). The protein profiles of crude enzymes were determined by SDS-PAGE (B). Lane no. 1-4 were crude proteases from TBRC 2902, TBRC 1180, TBRC 6663, and TBRC 7773, respectively, and lane 5 was Alcalase.

3.3 Effect of surfactants on protease activities

Alkaline proteases are employed as additives in detergents, and the stability of these proteases to surfactants is an important property for this industrial application. The effects of five surfactants on the protease activities of each crude enzyme were determined, with water serving as a control (Figure 6). Significant reductions in residual activity were

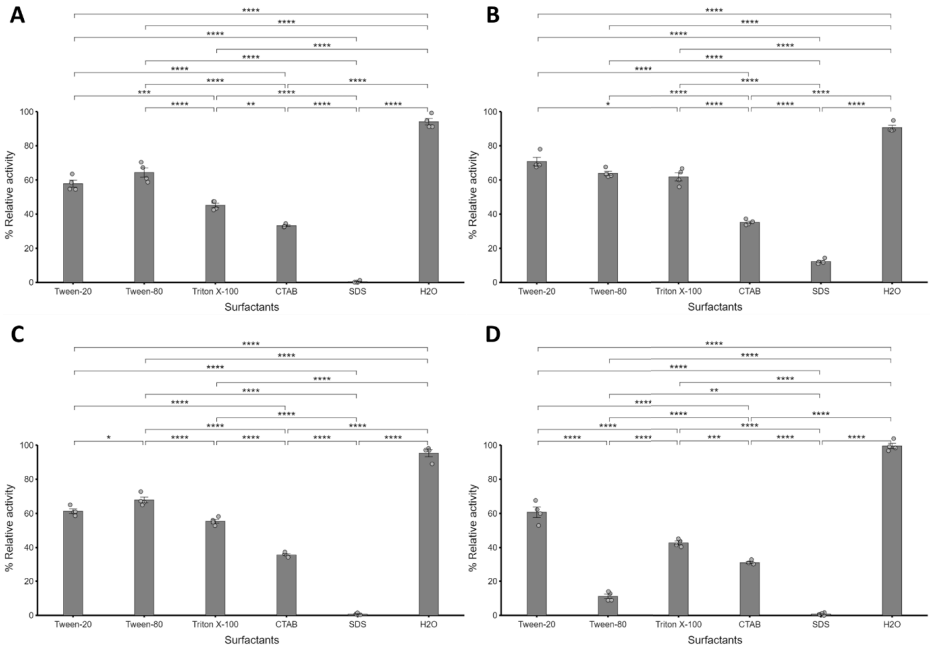


Figure 6. The effect of surfactants on protease activity in crude enzymes produced from *B. amyloliquefaciens* TBRC 2902 (A), *B. siamensis* TBRC 1180 (B), *B. subtilis* TBRC 6663 (C), and *B. velezensis* TBRC 7773 (D), using casein in 50 mM Tris-HCl buffer, at pH 9.0 as the substrate. The reaction mixtures were incubated at 40°C for 24 hours. Water at the starting time point (T_0) was used as a control. Asterisks represent statistically significant differences (p-value < 0.05).

observed for enzymes incubated with all surfactants, and significant differences were observed among surfactants for each enzyme. The most potent inhibitory effect was observed with SDS. *B. siamensis* TBRC 1180 enzyme was the most tolerant of SDS, with 12.15% relative activity remaining. By contrast, less than 1% of the relative activity remained for the other enzymes when exposed to 0.5% (v/v) SDS. The enzymes were more tolerant of CTAB and the non-ionic detergents (Triton X-100, Tween-20, and Tween-80). Less than 40% relative activity remained for all enzymes after CTAB exposure, whereas 59-73% relative activity remained after Tween-20 exposure. The proteases produced by TBRC 2902, TBRC 1180, and TBRC 6663 were similarly tolerant to Tween-80, with the remaining relative activity ranging from 65% to 70%. However, the TBRC 7773 enzyme was markedly less tolerant of Tween-80 with only 11% relative activity remaining. All tested enzymes exhibited moderate tolerance to 5% (v/v) Triton X-100 exposure, with remaining relative activities ranging from 43% to 64%.

4 Discussion

Bacillus spp. are known for their robust production of extracellular alkaline proteases. In this study, the extracellular alkaline proteases produced from four *Bacillus* strains were studied. To optimize the production of alkaline protease, the bacteria were cultured under different conditions. Yeast extract was identified as the optimal organic nitrogen source for the production of protease activity in all tested strains (Figure 1A-D), similar to previous reports on *B. stearothermophilus* (Karray et al., 2021) and *B. subtilis* ATCC 6633 (Chatterjee et al., 2015). In contrast, peptone was a much less effective organic nitrogen source, unlike that reported for production of alkaline protease activity in *B. pumilus* MP 27 and *Bacillus* sp. RGR-14 (Puri et al., 2002; Baweja et al., 2016). Other organic nitrogen sources, including skim milk for TBRC 7773 and soytone for TBRC 6663 were comparable to yeast extract for the production of alkaline protease activity, suggesting that the optimal organic nitrogen source is strain-specific. Alternative organic nitrogen sources effective for the production of protease activity in other *Bacillus* strains include beef extract for *B. amyloliquefaciens*, *B. licheniformis*, *B. megaterium* and *B. subtilis* (Boominadhan et al., 2009; Sarker et al., 2013), soybean meal for *B. cereus* MCM B-326 (Nilegaonkar et al., 2007), soybean meal for *B. subtilis* PCSIR-5 (Qazi et al., 2006), and soya meal mixed with wheat bran for *B. mojavensis* SA (Hammami et al., 2018).

Regarding the effect of inorganic nitrogen sources on alkaline protease activity, diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) boosted the protease activity of TBRC 2902 to 20.8% of the activity from the basal medium, whereas potassium nitrate (KNO_3) increased the activity of TBRC 1180 by 11.2%, similar to that previously reported for *B. licheniformis* MTCC NO. 7053 (Lakshmi et al., 2014). However, none of the five tested inorganic nitrogen sources in this study were able to enhance the protease activity of TBRC 6663 and TBRC 7773. The activity of these two strains might be induced by other inorganic nitrogen sources as previously reported. For instance, the protease activity of *B. licheniformis* P003 in the basal medium was as high as

the activity in the basal medium supplemented with ammonium sulphate ($(NH_4)_2SO_4$) (Sarker et al., 2013). Moreover, ammonium sulphate could optimize the protease activity of *B. amyloliquefaciens* SH-2 (Boominadhan et al., 2009), *B. halodurans* (Balachandran et al., 2021), and *B. licheniformis* SW2 (Boominadhan et al., 2009), while ammonium carbonate ($(NH_4)_2CO_3$) was essential for protease activity produced by *B. subtilis* DL-1 and *B. megatherium* MW-1 (Boominadhan et al., 2009). *B. subtilis* EFRL 01 (Qureshi et al., 2011) and *B. subtilis* IC-5 (Gul et al., 2015) preferred ammonium nitrate (NH_4NO_3) and sodium nitrate ($NaNO_3$) as inorganic nitrogen sources, respectively.

In addition to the nitrogen source, the carbon source is important for the production of extracellular alkaline protease. Tapioca starch was identified as the optimal carbon source for enzyme production in TBRC 1180 and TBRC 2902, whereas the optimal carbon sources for TBRC 6663 and TBRC 7773 were soluble starch and sucrose, respectively. The ability of TBRC 1180 and TBRC 2902 to optimally produce protease using tapioca starch, an inexpensive carbon source, makes these strains potentially advantageous for industrial application over other *Bacillus* strains that require more expensive simple carbon sources, such as glucose for *B. luteus* H11 (Kalwasińska et al., 2018) and *B. subtilis* IC-5 (Gul et al., 2015), and maltose and fructose for *B. horikoshii* (Joo and Choi, 2012). Nonetheless, strain-specific preferences for other inexpensive carbon sources have been reported, including rice flour for *B. licheniformis* P003 (Sarker et al., 2013), wheat straw for *B. pumilus* MP 27 (Baweja et al., 2016) and *Bacillus* sp. BBXS-2 (Qureshi et al., 2016), and molasses for *Bacillus* sp. BGS (Moorthy and Baskar, 2013), *B. subtilis* (Helal et al., 2012), and *B. pantotheneticus* (Shikha et al., 2007).

Regarding the effect of culture incubation time on the production of protease activity, TBRC 1180 and TBRC 6663 produced protease in a short time, with peak activity observed after 24 hours of culture, similar to *B. stearothermophilus* (Karray et al., 2021). However, TBRC 2902 and TBRC 7773 required a longer culture period (48 hours) to reach peak production, similar to *B. licheniformis* P003 (Sarker et al., 2013), *B. pantotheneticus* (Shikha et al., 2007), *B. pumilus* MP 27 (Baweja et al., 2016), *B. subtilis* GA CAS8 (Sathishkumar

Table 1. Comparison of physicochemical properties of proteases from various *Bacillus* spp. with the crude proteases from four *Bacillus* strains in this study

Organism	Type	MW (kDa)	Optimal pH	Optimal temperature (°C)	Reference
B. altitudinis W3	PE	34.9	10.5	45	Yang et al., 2020b
B. altitudinis W3	PE	37.3	8.5	50	Yang et al., 2020b
B. altitudinis W3	PE	37.9	9.5	55	Yang et al., 2020b
B. alveayuensis CAS 5	PE	33	9	50	Annamalai et al., 2014
***B. amyloliquefaciens* TBRC 2902**	**CE**	**n.d.**	**8**	**50**	**This study**
B. amyloliquefaciens SYB-001	PE	36.8	7	50	Wang et al., 2013
B. caseinilyticus	PE	66	8	60	Mothe and Sultanpuram, 2016
B. cereus VITSN0	CE	32	8	30	Sundararajan et al., 2011
B. gibsonii 6BS15-4	CE	**n.d.**	12	60	Mahakhan et al., 2023
B. invictae AH1	CE	**n.d.**	9.0 - 11.0	60	Hammami et al., 2017
B. koreensis BK-P21A	PE	48	9	60	Anbu, 2013
B. licheniformis ALW	PE	**n.d.**	9	70	Emran et al., 2020
B. licheniformis P003	CE	**n.d.**	10	50	Sarker et al., 2013
B. licheniformis RSP-09-37	PE	55	10	55	Sareen and Mishra, 2008
B. luteus H11	PE	37	10.5	45	Kalwasińska et al., 2018
B. megaterium	PE	25	7.5	50	Asker et al., 2013
B. megaterium	PE	28	7.5	50	Asker et al., 2013
B. megaterium	PE	33	8	70	Manavalan et al., 2020
B. safensis S406	PE	29	11	60	Mhamdi et al., 2017
***B. siamensis* TBRC 1180**	**CE**	**n.d.**	**9**	**60**	**This study**
Bacillus sp.SM2014	PE	71	10	60	Jain et al., 2012
***B. subtilis* TBRC 6663**	**CE**	**n.d.**	**8**	**50**	**This study**
B. subtilis BP-36	PE	40	9	60	Mashayekhi et al., 2012
B. subtilis DR8806	PE	37	8	45	Farhadian et al., 2015
B. subtilis GA CAS	PE	41	9	50	Sathishkumar et al., 2015
B. thuringiensis	PE	**n.d.**	8	47	Agasthya et al., 2013
***B. velezensis* TBRC 7773**	**CE**	**n.d.**	**8**	**50**	**This study**
B. velezensis SW5	PE	34	8	40	Yang et al., 2020a

Note: Remark: CE, crude enzyme; PE, purified enzyme; n.d. not determined.

et al., 2015), *B. subtilis* (Helal et al., 2012), and *Bacillus* sp. BGS (Moorthy and Baskar, 2013). Prolonging the incubation time beyond 48 hours can lead to a decrease in protease activity (Karray et al., 2021).

When used as an additive in detergent, proteases with optimal activity and stability under alkaline conditions are desirable. In this study, the crude enzymes from all tested strains showed protease activity with optimal activity in alkaline conditions. *B. amyloliquefaciens* TBRC 2902, *B. subtilis* TBRC 6663, and *B. velezensis* TBRC 7773 showed optimal activity at pH 8.0, similar to the crude enzyme produced from *B. cereus* VITSN04 (Sundararajan et al., 2011), and the purified enzymes from *B. caseinilyticus* (Mothe and Sultanpuram, 2016), *B. megaterium* (Manavalan et al., 2020), *B. subtilis* DR8806 (Farhadian et al., 2015), *B. thuringiensis* (Agasthya et al., 2013), and *B. velezensis* SW5 (Yang et al., 2020a). By contrast, *B. siamensis* TBRC 1180 exhibited optimal activity at pH 9.0, similar to the purified enzymes produced from *B. alveavuensis* CAS 5 (Annamalai et al., 2014), *B. koreensis* BKP21A (Anbu, 2013), *B. licheniformis* ALW (Emran et al., 2020), and *B. subtilis* BP-36 (Mashayekhi, 2012) (Table 1).

Proteases generally exhibit optimal activity in the range 50 to 70°C (Haddar et al., 2009a, b). The crude enzymes from TBRC 2902, TBRC 6663, and TBRC 7773, showed optimal activity at 50°C, similar to that of the crude enzyme from *B. licheniformis* P003 (Sarker et al., 2013), and the purified enzyme from *B. altitudinis* W3 (Yang et al., 2020b), *B. alveayuensis* CAS 5 (Annamalai et al., 2014), *B. amyloliquefaciens* SYB-001 (Wang et al., 2013), *B. megaterium* (Asker et al., 2013), and *B. subtilis* GA CAS8 (Sathishkumar et al., 2015). By contrast, the TBRC 1180 crude enzyme exhibited optimal activity at 60°C, similar to the crude enzymes of *B. gibsonii* 6BS15-4 (Mahakhan et al., 2023), and *B. invictae* AH1 (Hammami et al., 2017), and the purified enzymes of *B. caseinilyticus* (Mothe and Sultanpuram, 2016), *B. koreensis* BK-P21A (Anbu, 2013), *B. safensis* S406 (Mhamdi et al., 2017), and *B. subtilis* BP-36 (Mashayekhi, 2012) (Table 1).

The stability of alkaline proteases to surfactants is important for industrial application in detergent formulations, which was investigated using non-ionic and ionic surfactants for the crude enzymes. Regarding the non-ionic surfactants, the crude enzymes from TBRC 1180 and TBRC 6663 exhibited moderate stability in 5% (v/v) Triton X-100, showing relative activities of 63.41±5.18% and 56.90±3.47%, respectively, after 24 hours of incubation. These values were higher than the relative activity of the crude enzymes from *B. licheniformis* A10, *B. invictae* AH1, and *B. mojavensis* SA, which was 44.38%, 46.83%, and 48.54%, respectively, after 1 hour of incubation (Yilmaz et al., 2016; Hammami et al., 2017, 2018). The relative activities of the crude enzymes from all tested strains against 5% (v/v) Tween 20 were above 45%, surpassing the residual activity of the partially purified enzymes from *B. licheniformis* A10, and *Bacillus* sp. RGR-14, which were 34.29% and 35.00% after 1 of incubation at 5% and 1% (v/v) of the surfactant, respectively (Oberoi et al., 2001; Yilmaz et al., 2016). Furthermore, the crude enzyme of *B. subtilis* TBRC 6663 exhibited the highest relative activity against 5% (v/v) Tween 80, reaching 61.35±3.39%. This activity level was comparable to the relative activity of the purified enzymes from *Bacillus* sp. MPTK 712 and *B. licheniformis* A10, which were recorded at 67.50% and 27.84%, respectively (Kumar et al., 2012; Yilmaz et al., 2016).

In terms of activities on 1% (w/v) CTAB at 24 hours of incubation, TBRC 2902, TBRC 6663, and TBRC 7773 showed the relative activity of 30.64±0.62%, 32.25±1.13%, and 27.15±1.48%, respectively. These activities surpassed the relative activity after 1 hour of the purified enzyme from *B. subtilis* DR8806 at the same concentration, which was recorded at 25% (Farhadian et al., 2015). Among the tested enzymes, only the crude enzyme from TBRC 1180 maintained its activity in 0.5% (w/v) SDS after 24 hours, exhibiting a relative activity of 7.81±0.89%. This relative activity is comparable to the residual activity of *B. invictae* AH1, which was 20.54±0.83% after 1 hour of incubation with the same concentration of SDS (Hammami et al., 2017).

5 Conclusion

The extracellular alkaline proteases produced by *B. amyloliquefaciens* TBRC 2902, *B. siamensis* TBRC 1180, *B. subtilis* TBRC 6663, and *B. velezensis* TBRC 7773 were studied. The culture conditions for protease production were optimized. In addition, the biochemical properties of the crude enzymes were characterized. Among the tested enzymes, the crude enzyme from *B. velezensis* TBRC 7773 exhibited the highest specific activity, with optimal temperature and pH values of 50°C and 8.0, respectively. The other enzymes exhibited similar thermo-alkaliphilic properties, although the *B. siamensis* TBRC 1180 crude enzyme notably exhibited optimal temperature and pH values of 60°C and 9.0, respectively. All crude enzymes showed greater tolerance to non-ionic surfactants (Triton X-100, Tween 20, and Tween 80) than ionic surfactants, particularly SDS. The alkaline proteases produced by these *Bacillus* strains possess unique properties and versatility that make them well-suited for industrial applications.

Author Contributions

KP, VC, and TL conceived and designed the work. KP, KS, and TL performed the experiments, analyzed, and interpreted the data. WS and TL statistically analyzed the data. KB, KA, WS, and BB performed the preliminary experiment. KP, KS, and TL drafted the manuscript. TL, PK, and VC critically revised the manuscript for intellectual content. All authors have read and agreed to the submission of the manuscript.

Acknowledgments

This work was financially supported by National Science and Technology Development Agency (P-23-51623). The authors

would like to thank Dr. Philip James Shaw for manuscript proofreading, comments, and suggestions.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical Approval

Not applicable.

Data Availability Statement

The data underlying this article are available in the article.

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