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ORIGINAL PAPER

Production of *Myxococcus virescens* S2-2 Alkaline Protease by Utilizing Agro-Industrial Byproducts: A Sustainable Alternative for Detergent Additive and Chitin Extraction

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Abstract

Background Myxobacteria are Gram-negative bacteria renowned for their valuable production of secondary metabolites; however, their diverse protease enzymes remain less explored.

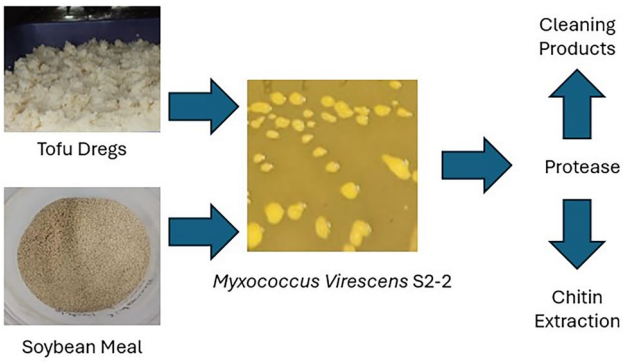
Aim In this investigation, a novel strain was isolated from mangrove soil that produces alkaline protease.

Methods Using 16S rRNA sequence analysis, the strain was identified as *Myxococcus virescens* species. The extracellular alkaline protease MvCP of strain S2-2 was produced using growth media containing agro-industrial by-products such as soybean meal and tofu dregs.

Results MvCP exhibited optimum activity within a pH range from 9 to 10 and temperatures ranging from 30 °C to 50 °C. MvCP activity was improved with Ca^{2+}, Mg^{2+}, and Zn^{2+} ions, whereas EDTA and β-mercaptoethanol at concentrations of 0.5 and 1.25 mM had no significant effect. MvCP was stable in chloroform, acetone, Triton X-100, Tween-20, and Tween 80.

Conclusion MvCP can be considered an eco-friendly option for use in household cleaning products and chitin extraction processes.

Graphical Abstract



Keywords Myxobacteria · Alkaline proteases · Agro-industrial byproducts · Soybean meal · Tofu dregs

Statement of Novelty

To the best of our knowledge, this is the first study to analyze and investigate the potential uses of alkaline proteases produced by *Myxococcus virescens*.

Extended author information available on the last page of the article

Introduction

Microorganisms are widely distributed in nature and are recognized as one of the most promising biochemical reservoirs due to their ability to produce diverse and multifunctional enzymes. These enzymes, known for their robustness, exhibit properties such as thermostability and pH tolerance, making them ideal candidates for industrial processes under varying physio-chemical conditions [1]. The demand for microbial enzymes has grown steadily, driven by the need for sustainable and efficient solutions in industries ranging from food and agriculture to chemicals and medicine. Microbial enzymes are particularly valued for their ability to reduce energy consumption and environmental impact while delivering cost-effective and eco-friendly biotechnological processes [2, 3].

Microbial proteases demonstrate a broad range of proteolytic activity across various pH and temperature levels, surpassing those derived from animal and plant sources. These enzymes are predominantly extracellular, secreted directly into the production media, which facilitates their separation from microbial cells through centrifugation or filtration, leaving the crude enzyme in the supernatant or filtrate. Alkaline proteases from microbial sources are particularly stable and can be produced in large quantities, making them highly valuable in numerous industries due to their exceptional activity and stability under alkaline conditions [4, 5].

Myxobacteria, part of the phylum Myxococcota [6], are rod-shaped, Gram-negative bacteria known for their unique life cycle and communal behavior. When nutrients become limited, many myxobacteria swarm together, aggregate, and form multicellular fruiting bodies [7–10]. These structures contain resilient, dormant myxospores that help ensure the survival of the species. Myxobacteria are commonly found in various environments such as topsoil, animal dung, decaying plant material, and tree bark [7–10], and they have also been discovered in salt-tolerant (halophilic) environments [11–14]. As mesophiles, they grow best at temperatures between 6 °C and 38 °C and can tolerate pH levels ranging from 5 to 9.

In recent decades, the isolation of myxobacteria has focused primarily on their potential in natural product discovery, particularly for finding new antibiotics, anticancer agents, and antiviral compounds [15–18]. Myxobacteria are known to produce a wide range of secondary metabolites with antibiotic properties, which help them kill prey, while their hydrolytic enzymes break down the macromolecules of their prey [19]. Due to their strong ability to degrade biomacromolecules, myxobacteria are classified as bacteriolytic and cellulolytic organisms [9]. For instance, *Myxococcus xanthus* produces lipases, esterases, and proteases with antimicrobial properties [20], while *Corallococcus coralloides* produces chitinase, which efficiently converts chitin into chitin-oligosaccharides [21]. Additionally, extracellular proteases from the S252 isolate (PyCP) have diverse applications, including use as detergent additives, agents for hydrolyzing gelatin and recovering silver from used radiographs, and as effective agents for de-furring animal hides [22].

Agro-industrial byproducts, derived from the processing of various crop plants, present a significant environmental challenge due to improper disposal, despite their rich nutrient content and industrial potential [23]. For instance, Indonesia's tofu industry produces approximately 1.024 million tons of tofu dregs annually, a protein- and fiber-rich waste product, yet struggles with waste management due to inadequate treatment facilities [24]. Similarly, soybean meal, a byproduct of oil extraction, contains around 50% protein and is utilized in animal feed or protein extraction for food applications [25]. Due to their composition, these byproducts can be utilized as a cost-effective and sustainable substrate for fermentation processes. Previous studies on *Bacillus cereus* found that using ternary waste as a cost-effective substrate led to the production of alkaline protease [4, 5].

The main goal of this study was to explore the potential biotechnological applications of proteases produced by a newly isolated strain of *Myxococcus virescens* S2-2, using agro-industrial byproducts like soybean meal and tofu dregs as substrates. To the best of our knowledge, this is the first study to analyze and investigate the potential uses of alkaline proteases produced by *Myxococcus virescens*.

Materials and Methods

Myxobacterial Strains and Culture Conditions

During this investigation, 11 myxobacterial strains were isolated from the soil sample collected in the mangrove area in Bali, Indonesia (8°43′32.3"S 115°13′37.0"E) in December 2022 and used for preliminary protease activity screening. Myxobacterial strain was isolated using the traditional bacterial baiting technique, employing live *E. coli* K-12 DSM 498 on water agar with soil samples, and then purified by repeatedly transferring the outer rim of the of the swarming colony to fresh mineral salt medium plates [26]. Pure culture was cultured at 30 °C on VY/2 agar [8–10] and CY–H medium [26].

Screening of Protease-Producing Strains

Myxobacterial strains were cultivated in CY medium with pH 7.2 and cultured for 5 days at 30 °C with shaking at 160 rpm. The supernatant was acquired through centrifugation at 8000 rpm for 15 min and utilized as crude

protease. The protease activity produced by the myxobacterial strains was assessed on skim milk agar [22] with pH 10.

Protease activity was measured by mixing 1% casein substrate with crude protease in equal volumes in a pH 12 solution (0.05 M KCl–NaOH) and then incubating the mixture for 1 h at 40 °C. The enzymatic reaction was stopped by the addition of 10% TCA (trichloroacetic acid), equivalent to double the initial reaction volume, followed by incubation for 30 min at 37 °C. Following a spin at 10,000 rpm for 10 min to separate the precipitate, of the supernatant (500 µL) was mixed with Na_2CO_3 (0.4 M; 2.5 mL) and Folin-Ciocalteu's phenol reagent (1 N; 250 µL) [27] and was incubated for 30 min at 37 °C. The absorbance of the mixture was determined at 660 nm utilising a spectrophotometer. A unit of enzymatic activity was quantified as the quantity of the protease needed to release 1 µg of tyrosine per minute per milliliter under standardized conditions. A standard curve for tyrosine was determined using L-tyrosine as a reference.

Strain Identification

The highest protease-producing strain was selected for further investigation. Genomic DNA extraction was conducted using cells harvested from actively growing CY–H cultures, adhering the standard method for Gram-negative bacteria with the Wizard® Genomic DNA Purification Kit (Promega) [28]. The 16S rRNA gene was amplified using universal primers F27 and R1492 [29]. PCR products were cleaned-up by employing Wizard® SV Gel and PCR Clean-Up System (Promega) [28], separated by gel electrophoresis (0.8% (w/v) agarose, at 80 V for 30 min), and afterward sequenced using the Sanger method with F27 and R1492 primers. Chromas software (version 2.6.6) was employed for correcting the 16S rRNA gene sequence. The NCBI BLAST tool (https://www.ncbi.nlm.nih.gov/) was utilized to identify closely related organisms based on the 16S rRNA gene sequence [30].

Protease Production with Various Media

To assess protease production under varying cultivation conditions, the selected strain was cultured in six distinct media formulations adjusted to pH 7.2 and cultured at 30 °C for 12 days with 160 rpm of agitation. The media compositions used for protease production included CY medium, CY + Glu medium (CY medium supplemented with 1% glucose), Tofu medium (containing 1.5% tofu dregs), Tofu + Glu medium (1.5% tofu dregs with 1% glucose), Soy medium (comprising 1.5% soybean meal), and Soy + Glu medium (1.5% soybean meal supplemented with 1% glucose). The crude protease was obtained by centrifugation at 8000 rpm for 15 min and proteolytic activity was determined using a protease activity assay with casein as the substrate.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weights of the crude proteases were assessed using SDS-PAGE comprising a stacking gel (4%) and resolving gel (10%) [31, 32]. Visualization of protein bands was achieved by staining with Coomassie Brilliant Blue [33]. The zymogram analysis of the crude protease was carried out following Sharma et al. [22].

Characterization of Crude Protease

pH Influence on the Activity and Stability of Proteases Generated by the Selected Strain

The influence of pH on the activity and stability of crude proteases was investigated over a pH range of 8.0 to 13.0. Enzyme assays were conducted using 1% casein in different pH (pH 7–13). The stability of the crude protease was assessed by preincubating in buffers (pH 4–12) at 4 °C for 16 h or in solutions from pH 7 to 12 for at 40 °C 1 h. Residual activity was resolved using a protease assay [22].

Influence of Temperature on the Enzymatic Activity and Stability of Proteases from the Selected Strain

The impact of temperature on the activity of crude proteases was investigated by conducting reactions within the range of 10 to 70 °C at the optimal pH for 1 h. The protease thermostability was examined by pre-incubating the crude protease at different temperatures (40–80 °C) for various durations (15–120 min). Residual activity was evaluated employing standard test protocols, with the untreated protease acting as the control (100% activity).

Impact of Metal Ions on Protease Activity

The crude proteases derived from the selected strain were exposed to varying concentrations (0.5 mM–2.5 mM) of Cu^{2+}, Ca^{2+}, $Mg^{2}+$, Zn^{2+}, Co^{2+}, and Ag^{+} at 40 °C for 60 min. After incubation, residual enzymatic activity was assessed using standard assay protocols, with the activity of the crude protease with no additional metal ions used as the control (100% activity).

Impact of Inhibitors on Protease Activity

The proteases produced by the selected strain were treated with 0.5 mM and 1.25 mM concentrations of ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol (β-ME) at

40 °C for 60 min. After incubation, residual enzyme activity was evaluated using standard assay procedures. A reaction mixture without inhibitors served as the control (100% activity).

Impact of Surfactants and Market-Available Detergents on Protease Activity

The impact of surfactants on crude proteases was evaluated applying sodium dodecyl sulfate (SDS) at concentrations of 0.5%, 1.25%, and 2.5% (w/v), as well as Triton X-100 and Tween 80 at 0.5%, 1.25%, and 2.5% (v/v). Additionally, the effects of solid commercial detergents (Attack, Rinso, Daia Putih at 0.4% w/v) and a liquid detergent (Rinso at 1:200 dilution) on crude proteases were investigated. The enzymes were subjected to denaturation by heating the solution for two hours. The crude proteases were pre-incubated with the surfactants or detergents for 1 h at 40 °C, and the residual enzyme activity was measured with Folin-Ciocalteu method. The residual activity of crude proteases incubated with no surfactant and detergent at 40 °C for 1 h served as the control (100% activity).

Impact of Organic Solvents on Protease Activity

The proteases derived from the selected strain were exposed to various organic solvents (25% v/v) including chloroform, acetone, butanol, ethanol, isopropanol, methanol, DMSO, and formamide at 40 °C for 1 h. Subsequently, protease activity was assessed using standard assay methods. The enzyme activity following incubation at 40 °C for 1 h in the absence of organic solvents was considered as 100%.

Substrate Specificity of the Proteases

Exploring substrate specificity in crude proteases involved assessing their activity on (1%) gelatin, casein, and bovine serum albumin (BSA). The proteases were incubated with various substrates at optimal temperature and pH conditions for 1 h to measure their relative activity.

Chitin Extraction from Shrimp Shells using Proteases

To assess the deproteinization of shrimp shells using MvCP, one gram of shrimp shells was rinsed thoroughly with water and pulverized using a pestle and mortar. The shells were subsequently exposed to MvCP at 40 °C (pH 8 and 12), followed by deactivation of the enzyme by incubating the reaction mixture at 85 °C for 1 h. After rinsing with distilled water, the shells were dried at 50 °C for 16 h. The protein concentration of the shells was evaluated before and after enzymatic treatment employing energy-dispersive X-ray spectroscopy (EDX; Oxford X-Max 20) to quantify the degree of deproteinization. The deproteinization percentage (DP%) was determined by the nitrogen-to-protein conversion factor 6.25 [34] and the equation provided by Rao et al. [35].

$$DP\% = \frac{(Pc\ .\ C) - (Pt\ .T)}{(Pc\ .\ C)} * 100$$

where Pc and Pt are the protein concentrations (%) before and after enzymatic treatment, respectively, and C and T are the dry weight masses of original and hydrolyzed sample, respectively.

Fourier Transform Infrared Spectroscopy

The functional groups of chitin were analyzed via Fourier transform infrared (FTIR) spectroscopy on a Nicolet i5 instrument. Spectral examination was performed from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} [36].

Scanning Electron Microscopy Analysis

The surface properties of the deproteinized shrimp shell was investigated using a scanning electron microscope (SEM; IT-300, JEOL) operating at an acceleration voltage of 10 kV. Before analysis, the shrimp shell samples were coated with a layer of gold.

Statistical Analysis

Each experiment was conducted in triplicate, and the data are expressed as mean ± standard deviation. Statistical significance difference was evaluated using Student's t-test.

Results

Screening of Protease-Producing Strains, Strain Identification, and SDS-PAGE Analysis

All 11 isolates were subjected to protease activity screening to identify the most proficient protease-producing myxobacterial strain. After screening all strains on skim milk agar at pH 10, the strains with the largest inhibition zones were selected for further analysis (data not shown). Among them, strain S2-2 showed a 19 mm clear zone. The assay using 1% casein revealed that each isolate demonstrated protease production, with two isolates, namely S2-2 and S7-22, exhibiting protease activity exceeding 50 units/ml (Fig. 1). Strain S2-2 was chosen as the best protease-producing strain since it had the highest protease activity of all examined strains

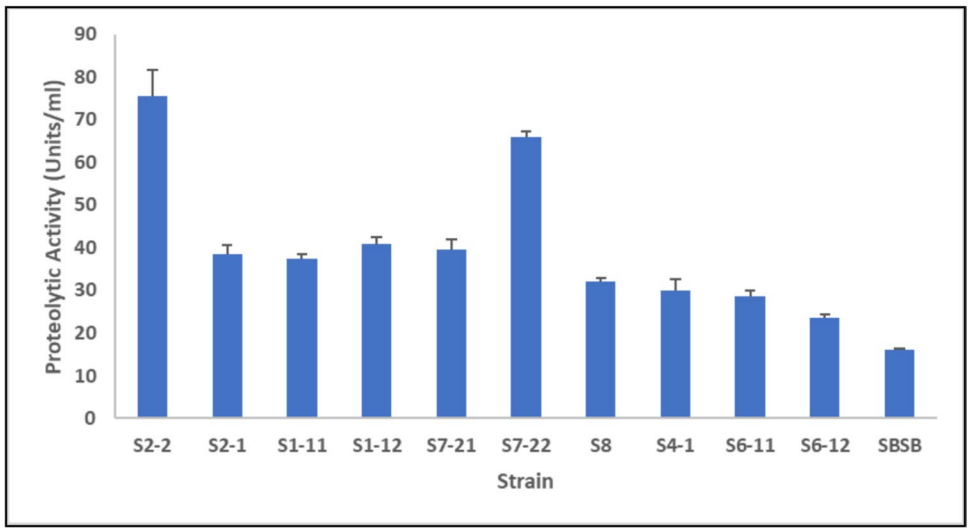


Fig. 1 Protease activity of 11 myxobacterial strains

($p < 0.1$ with S7-22 and $p < 0.05$ with the other strains) and was further studied including 16S rRNA gene analysis.

The almost-complete 16S rRNA gene sequences of strain S2-2 (1361 nt, GenBank accession number (PP789695) was determined and compared with the 16S rRNA gene sequences retrieved from EZBioCloud server (https://www.ezbiocloud.net/). Strain S2-2 was most closely related to *Myxococcus virescens* NBRC 100334 ^{T} (100%), *Myxococcus xanthus* DSM 16526 ^{T} (99.93%), and *Myxococcus vastator* AM301^{T} (99.71%).

SDS-PAGE analysis of *Myxococcus virescens* strain S2-2 crude proteases (MvCP) revealed major bands with molecular weights of around 50 kDa, 20 kDa, and less than 18 kDa. Zymogram of the crude proteases indicated that the proteolytic activity could be found in band ranging from around 50 to 200 kDa as depicted in Fig. 2.

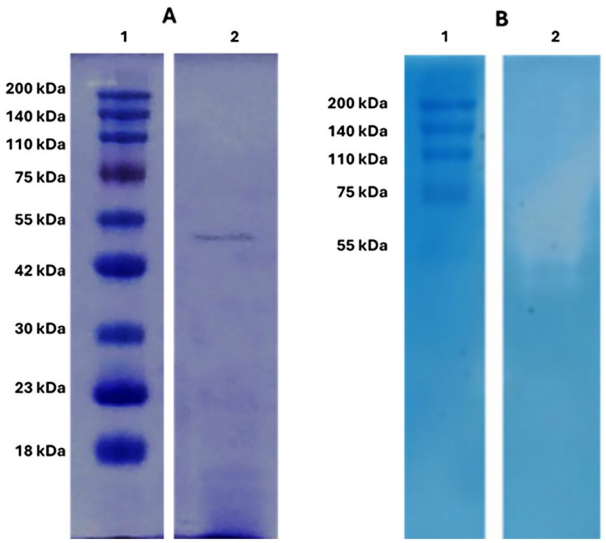


Fig. 2 Analysis of SDS-PAGE (**A**) and zymogram (**B**) for the extracellular protease produced by strain S2-2. Lane 1, prestained protein marker; Lane 2, MvCP

Influence of Different Medium on Protease Production

Evaluation of the effect of various medium used for protease production was carried out using six different media (CY, CY + Glu, Tofu, Tofu + Glu, Soy, and Soy + Glu). As can be seen in Fig. 3, among the media tested, Soy + Glu medium supported the maximum proteolytic activity after 192 h (8 days) (8 days) ($p < 0.05$).

Effect of pH on the Activity and Stability

As depicted in Fig. 4, proteolytic activity increased with pH, reaching the greatest value in a phosphate buffer at pH 10, relatively high at pH 11–12, and then rapidly decreased to a minimum value at pH 13. Proteolytic activity at pH 10 was significantly higher than the activity at pH 12 ($p < 0.05$).

The stability of MvCP at diverse pH levels was assessed by incubating the enzyme beforehand at 4 °C for 16 h and at 40 °C for 1 h in various buffer solutions. MvCP demonstrated optimal stability at pH 8, showing a significant difference compared to pH 7 ($p < 0.05$) and pH 9 ($p < 0.1$). The enzyme maintained over 70% of its activity after 16 h of incubation at 4 °C across a pH range of 5–9 (Fig. 5). Following 1 h of incubation at 40 °C, MvCP demonstrated peak activity at pH 8, closely followed by 98% residual activity at pH 9. The difference in activity between pH 8 and pH 9 was not statistically significant ($p > 0.1$).

Impact of Temperature on Activity and Stability

MvCP was highly active (> 80%) at temperatures ranging from 30 to 50 °C and displayed the greatest activity at 40 °C ($p < 0.05$ with all tested temperature) (Fig. 6). At 20 °C and 60 °C, MvCP demonstrated 49% and 42% relative activity, respectively.

MvCP protease remained highly stable at 40 °C for up to 15 min of incubation (Fig. 7), with no significant difference in stability observed after 30 and 45 min ($p > 0.05$). After 120 min at 40 °C, MvCP retained 86% of its starting activity. However, at 80 °C, the residual activity significantly dropped to 7% after just 15 min.

The Impact of Metal Ions on MvCP Protease Generated by Isolate S2-2

The influence of several metal ions with different concentrations on MvCP activity was examined. MvCP relative activity elevated by approximately 160% in conditions consisting of 0.5 mM Ag^+ and 2.5 mM Ca^{2+} (Fig. 8). The presence of 1.25–2.5 mM Zn^{2+} and 2.5 mM Mg^{2+} enhanced MvCP relative activity by around 140%, although the effect of 0.5 mM Zn was not significantly different to that of 1.25 and 2.5 mM Zn^{2+} ($p > 0.1$). However, it was discovered that an increase of Ag^+ and Cu^{2+} concentration led to a reduction in MvCP relative activity.

The Influence of Inhibitors and Surfactants on MvCP produced by Isolate S2-2

The impact of inhibitors on MvCP activity was investigated using EDTA and β-mercaptoethanol. As illustrated in Fig. 9, neither EDTA nor β-mercaptoethanol significantly affected MvCP activity at concentrations of 0.5 and 1.25 mM. However, there was a significant difference ($p < 0.05$) in the effect of 0.5 mM and 1.25 mM EDTA.

MvCP produced by strain S2-2 demonstrated high resilience in the presence of 0.5 mM Triton X-100 and 0.5–2.5 mM Tween-20 (Fig. 10). The enzyme exhibited the highest relative activity (184%) with the addition of 2.5 mM Tween 80 ($p < 0.05$), while the lowest relative activity was detected when 2.5 mM SDS was present.

Resilience of Proteases Produced by Isolate S2-2 in the Presence of Organic Solvents

Evaluation of MvCP produced by isolate S2-2 was carried out towards various organic solvents. As depicted in Fig. 11, MvCP was most stable in chloroform (82%) and relatively stable in acetone (72%), albeit the stability of MvCP in both solvents was not significantly different ($p > 0.05$). The lowest stability of MvCP was found after the addition of formaldehyde (8%).

Substrate Specificity and Compatibility with Commercial Detergents

The substrate specificity study of MvCP produced by strain S2-2 revealed that casein was the most preferred substrate ($p < 0.05$), with BSA and gelatine as subsequent preferences, which exhibited relative activity levels of 76% and 18%, respectively, as shown Fig. 12.

MvCP was exceptionally stable in solid detergents Daia Putih and Attack, maintaining about 100% of its starting activity, whereas liquid and solid Rinso decreased slightly the activity of MvCP to 78% and 85%, respectively Fig. 13. The relative activity in liquid Rinso and in Daia Putih were significantly different ($p < 0.1$).

Extraction of Chitin through Deproteinization of Crustacean Shells

Proteins are tightly bound to the chitin exoskeleton of shrimp shells, necessitating deproteinization during chitin extraction. MyCP activity on shrimp shells, with an enzyme/substrate ratio (E/S) of 157.15 U/mg, achieved 50.88% deproteinization after 1 h at pH 8. At pH 12, the deproteinization was 44.98% after the same duration. Fourier-transform infrared spectroscopy (FTIR) examination of the deproteinized shrimp shells revealed characteristic chitin bands, while sustaining the carbohydrate structure as presented in Fig. 14. The FTIR spectrum revealed bands at 1652 cm^{-1}, 1558 cm^{-1}, and 1373–1375 cm^{-1}, relating to amide I, amide II, and amide III, respectively. Additionally, IR peaks at 3253.3–3256.6 cm^{-1} and 3087.5–3093.3 cm^{-1} were assigned to the vibrations of hydroxyl (– OH) and methyl ester ($-CO-CH_3$) groups, respectively. The deproteinization of shrimp shells using MvCP significantly altered the shell surface, as evidenced by scanning electron micrographs. Initially, the surface of the shells appeared smooth and undamaged, but post-MvCP treatment, it exhibited noticeable damage (Fig. 15). This indicates that MvCP treatment caused a disturbance in the dense structure of shrimp shells, likely due to the extraction of proteins.

Discussion

Myxococcus virescens strain S2-2, isolated from mangrove soil, exhibited strong proteolytic activity, and the produced protease using byproducts such as soybean meal and tofu dregs was characterized. *Myxococcus* and *Corallococcus* are typically found in various kinds of soil types [26, 37]. Proteases produced by myxobacterial strains have been

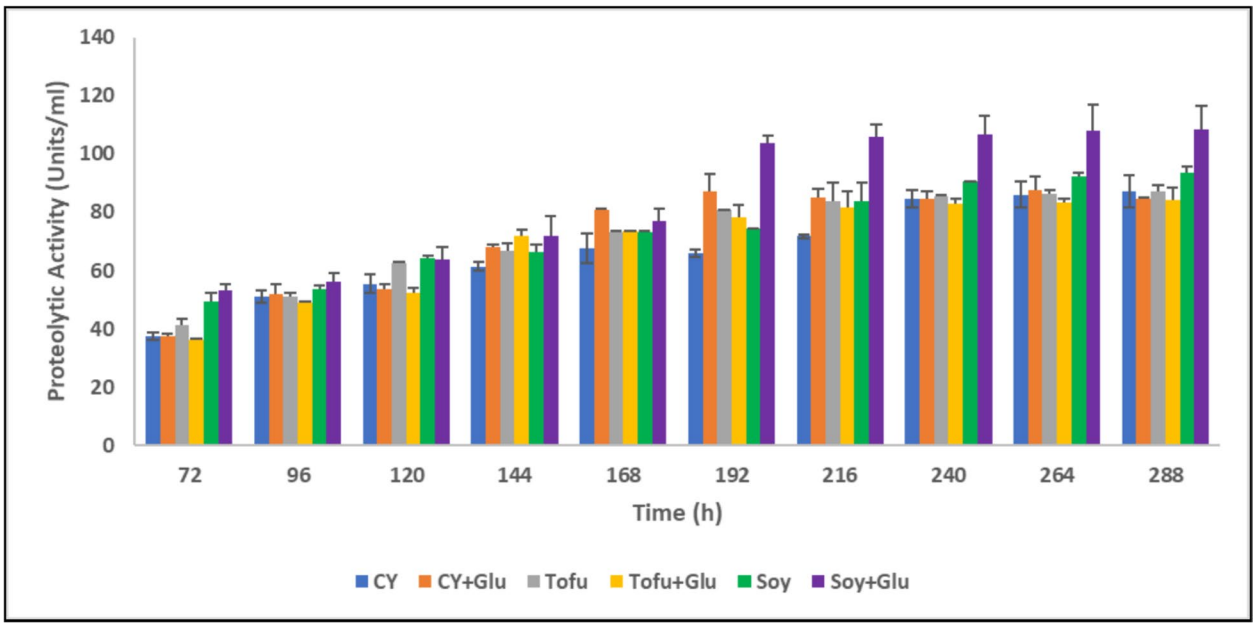


Fig. 3 Effect of different media on protease production

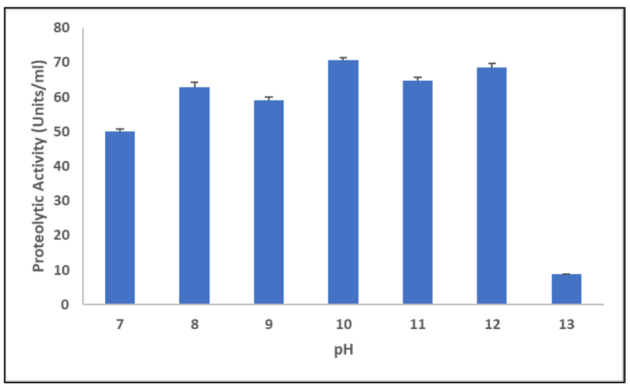


Fig. 4 pH optimum of MvCP protease

reported in some genera including *Myxococcus* [38–40], *Archangium* [41], and *Pyxidicoccus* [22]. Numerous myxobacteria produce proteases that may breakdown casein or hemoglobin [42].

Utilizing tofu dregs or soybean meal in the growth medium of *Myxococcus virescens* strain S2-2 facilitated the production of protease (MvCP), particularly when combined with glucose, resulting in optimal protease activity. Microbial protease production using soybean meal has also been documented in *Bacillus* [43] and *Aspergillus* [44]. Soybean meal serves as a valuable plant protein source, containing a crude protein concentration ranging from 440 to 530 g/kg. Its amino acid composition supports rapid digestion. Despite its low crude fiber content (31–72 g/kg), soybean meal is rich in phosphorus (6.3–6.4 g/kg), predominantly in the form of phytic acid [45].

MvCP showed both enzymatic activity and stability at alkaline pH levels, indicating its classification as an alkaline protease. These enzymes typically function within neutral to alkaline pH conditions. They can feature either a serine center, known as serine proteases, or belong to the metallo-type category, termed metalloproteases. Alkaline serine proteases, in particular, are highly valuable enzymes widely utilized across diverse industries globally [46].

Moderate heat stability of MvCP was determined with its highly activity (> 80%) at temperatures ranging from 30 to 50 °C. MvCP preserved more than 80% of the activity after 120 min of incubation at 40 °C. Alkaline protease produced from another myxobacterial strain, *Pyxidicoccus* sp. 252, was also reported to have optimal temperatures of 40–50 °C [22]. Another report indicated that the best temperature for alkaline protease from *Bacillus horikoshii* was 45 °C [47].

It was found that MvCP relative activity elevated with the present of Ca^{2+} (2.5 mM), Zn^{2+} (1.25–2.5 mM), and Mg^{2+} (2.5 mM). An increase of Ag^{+} and Cu^{2+} concentration resulted in a reduction on MvCP relative activity. The increased activity of protease produced by *Pyxidicoccus* sp. 252 increased in the presence of Mg^{2+} and Ca^{2+} ions [22]. Metal ions influence the catalytic activity and stability of proteases. Ca^{2+} and Mg^{2+} cations promote the contact between the catalytic region of protease and the substrate. Meanwhile, Cu^{2+} disrupt the catalytic site of the enzyme, resulting in a reduction of proteolytic activity [48].

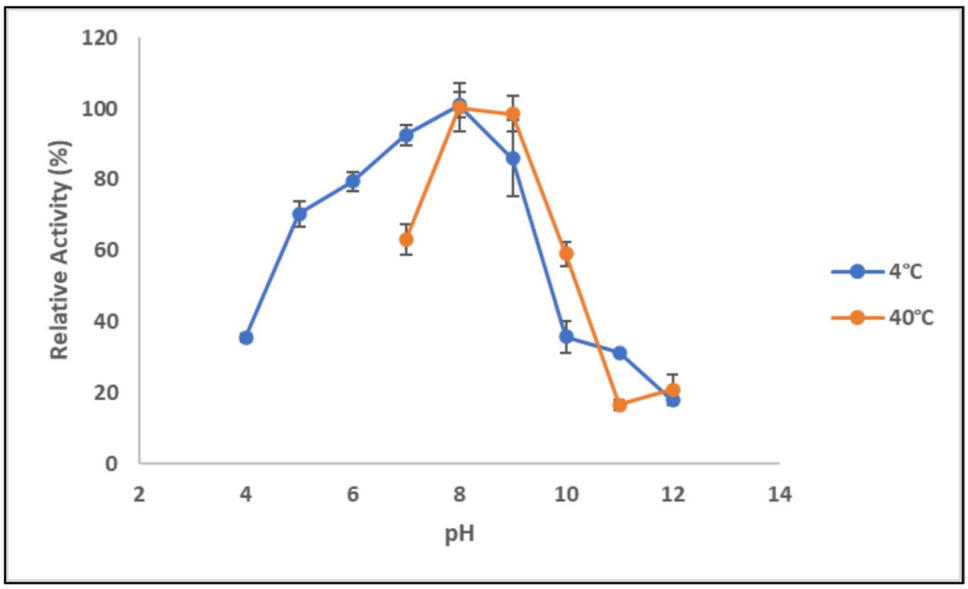


Fig. 5 pH stability of MvCP protease after pre-incubated in different temperatures

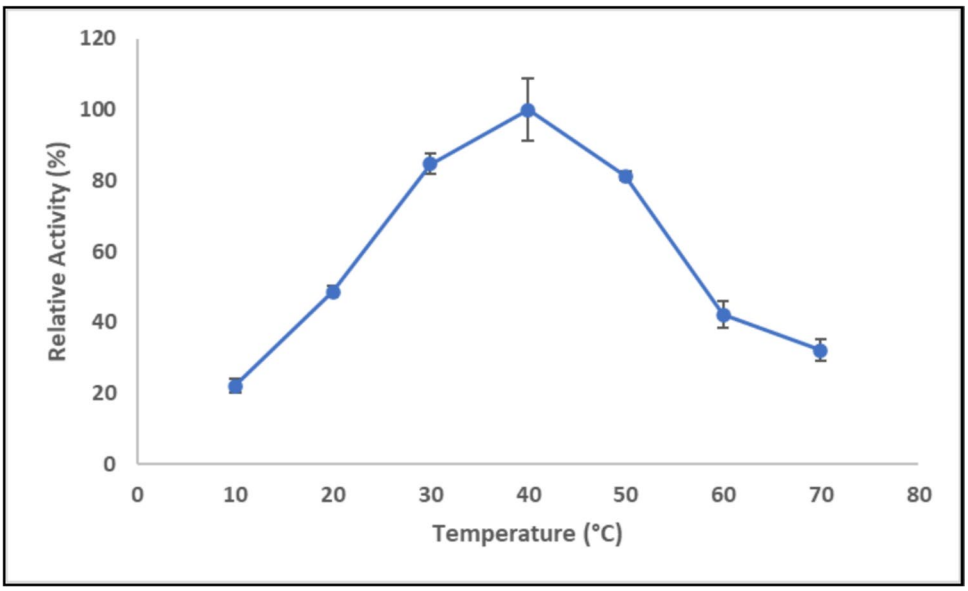


Fig. 6 Temperature optimum of MvCP protease

It was found that EDTA and β-mercaptoethanol at the concentration of 0.5 and 1.25 mM did not demonstrate any substantial effect on the activity of MvCP. Previous study showed that protease produced by *Bacillus* sp. EBTA6 decreased to around 50% with the addition of 1 mM EDTA [49]. Similarly, Saminathan and Narayanan [50], showed that 1 mM of EDTA significantly reduced the protease activity of *Bacillus subtilis* IAS 01 by 94.2%. In accordance with a comparable study described by Asitok et al. [51], the protease activity from *Stenotrophomonas acidaminiphila* was not significantly inhibited by β-mercaptoethanol at 5 mM concentrations.

The MvCP stability in various organic solvents was relatively high in chloroform and acetone, while it exhibited very low stability in formaldehyde. Karray et al. [52] also reported the presence of a chloroform-tolerant alkaline protease in *Bacillus stearothermophilus*. In the other study, the protease produced by *Bacillus subtilis* B22 was known to be relatively stable with acetone, hexadecane, and chloroform [48]. The very low stability of protease in formaldehyde was

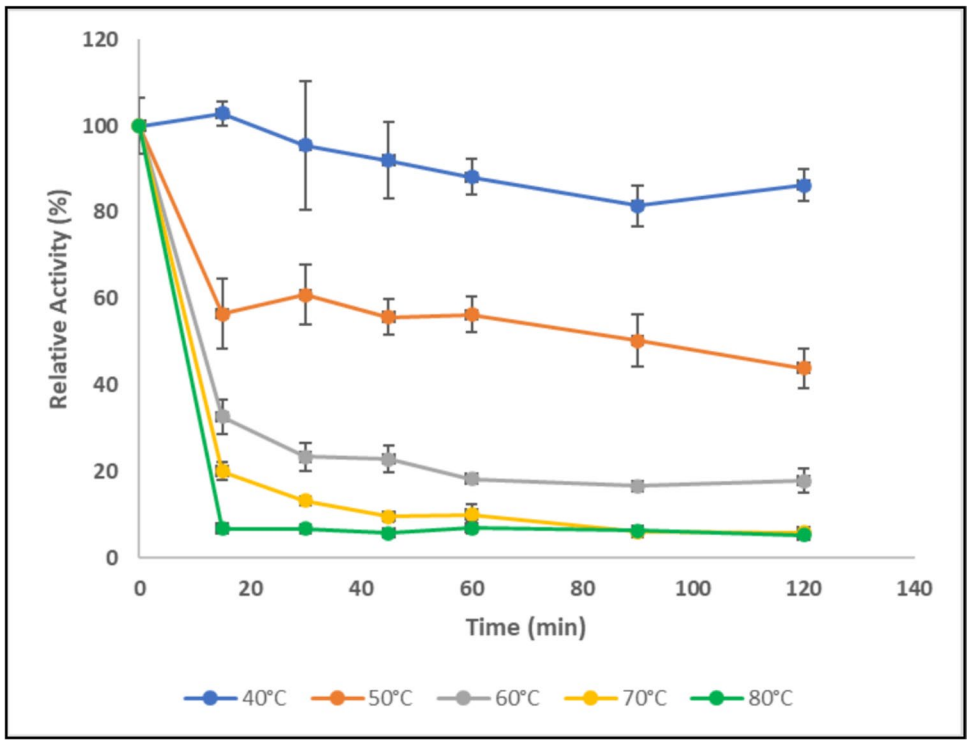


Fig. 7 Temperature stability of MvCP protease

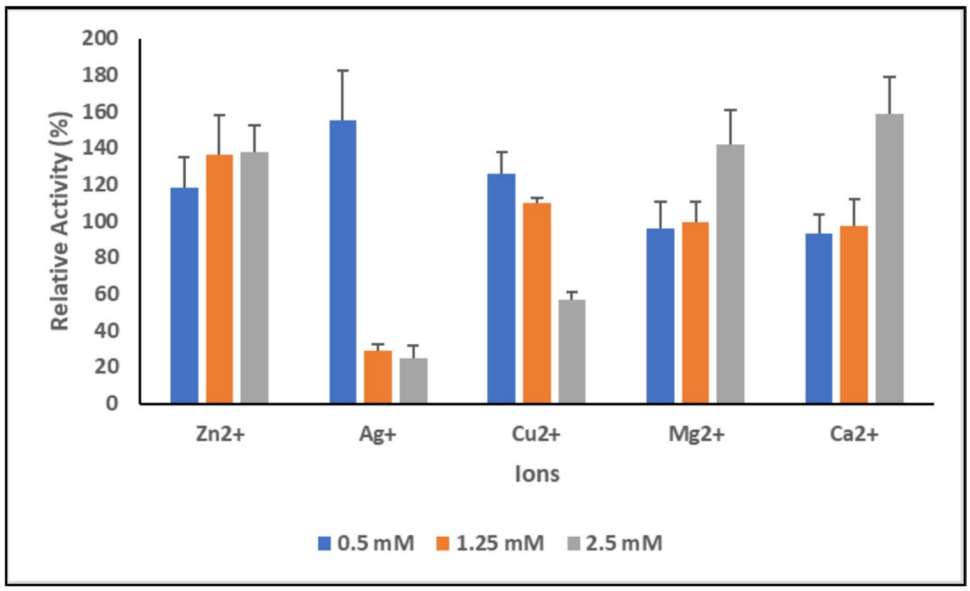


Fig. 8 Effect of metal ions on MvCP activity

observed in the study conducted by Sharma et al. [22] using *Pyxidicoccus* sp. 252 protease.

MvCP produced by strain S2-2 was highly stable in the presence of non-ionic surfactants such as Tween 20, Tween 80, and Triton X-100, whereas it showed reduced activity in anionic surfactant like SDS. In the same context, protease from *Bacillus swezeyi* B2 was also substantially active with the addition of non-ionic surfactant [53]. The poor activity of MvCP in the presence of SDS may be attributed to SDS denaturing effect on proteins [54]. Furthermore, the stability of MvCP in certain commercial detergents suggests its potential use as an ingredient in local household cleaning products.

The deproteinization study of MyCP on shrimp shells highlighted its potential for chitin extraction. Utilizing

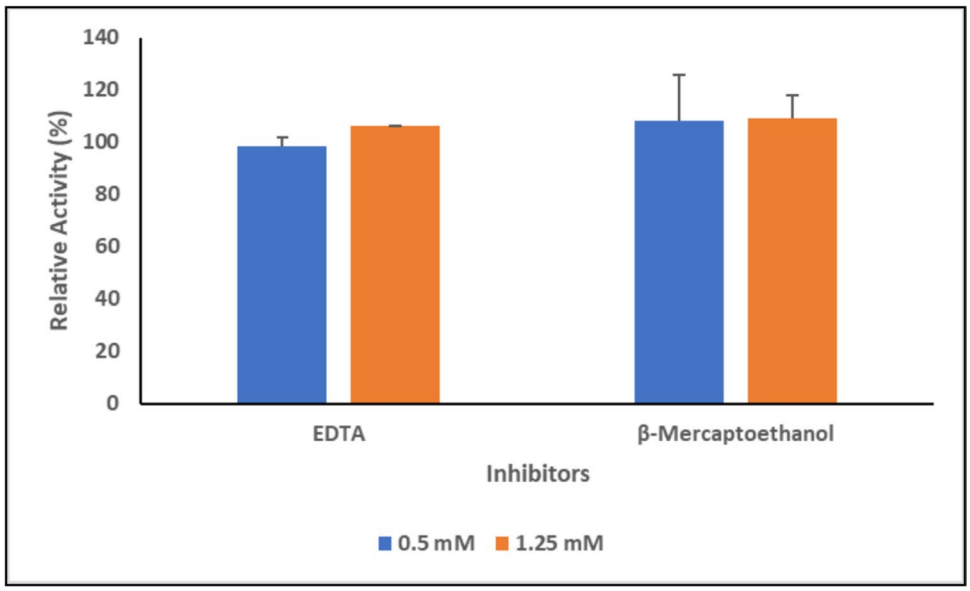


Fig. 9 Effect of inhibitors on MvCP activity

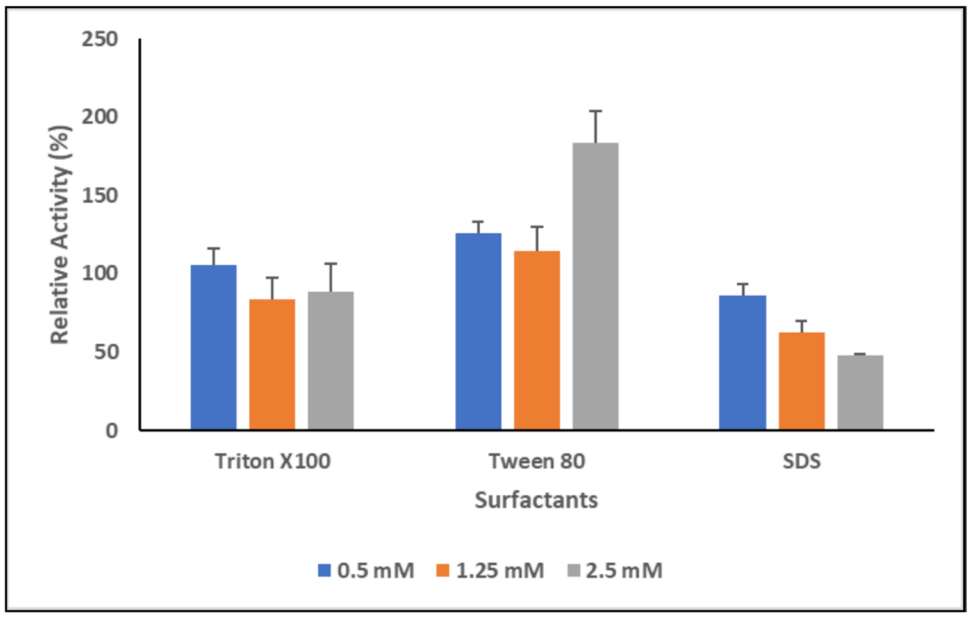


Fig. 10 Stability of MvCP in the presence of surfactants

protease-producing microorganisms for chitin recovery from shrimp shells offers a simple and sustainable method to valorize shrimp shell waste. For instance, Cahyaningtyas et al. [55] employed *Bacillus cereus* HMRSC30 protease to extract chitin from shrimp shells through an environmentally friendly process. Additionally, with the novel technique combining protease from *Bacillus subtilis* and microwave, chitin and chitosan were effectively produced from shrimp shells [56].

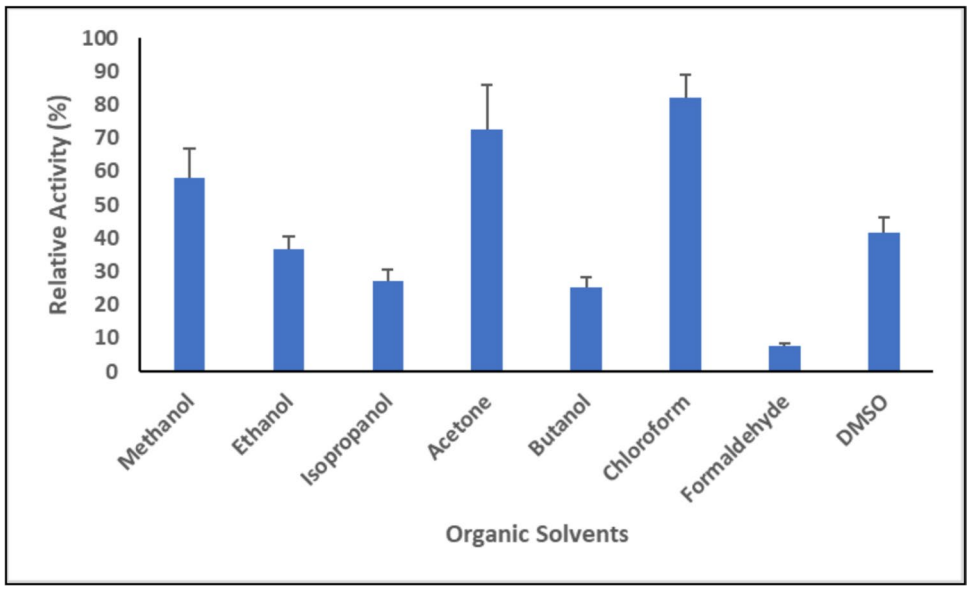


Fig. 11 Impact of organic solvents on MvCP enzymatic activity

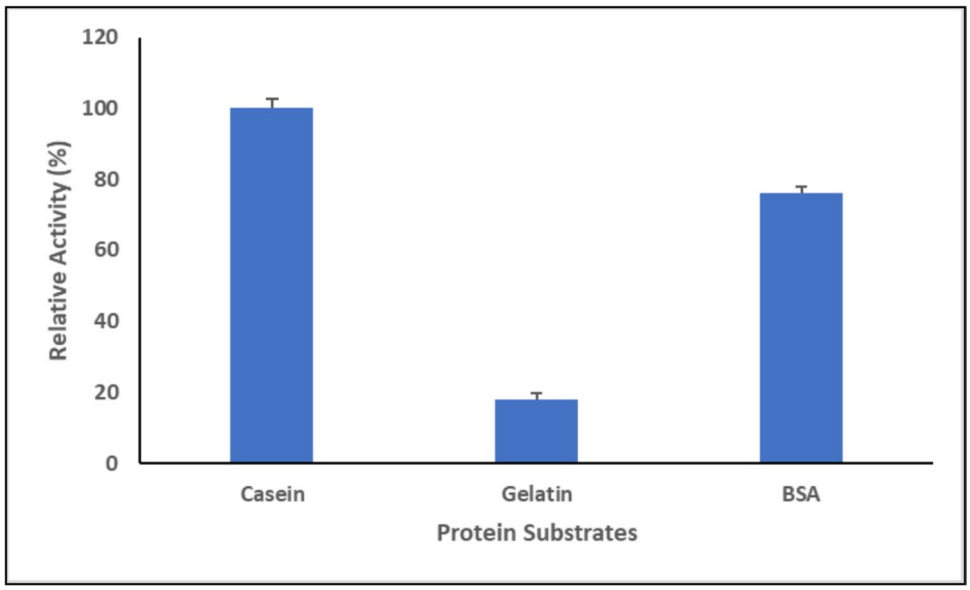


Fig. 12 MvCP substrate specificity

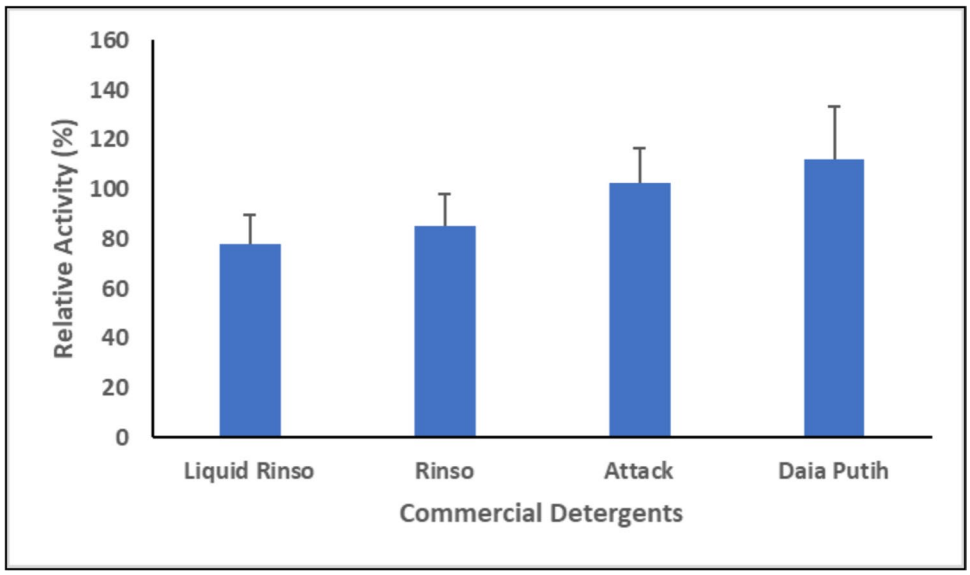


Fig. 13 Commercial detergents and their effect on MvCP stability

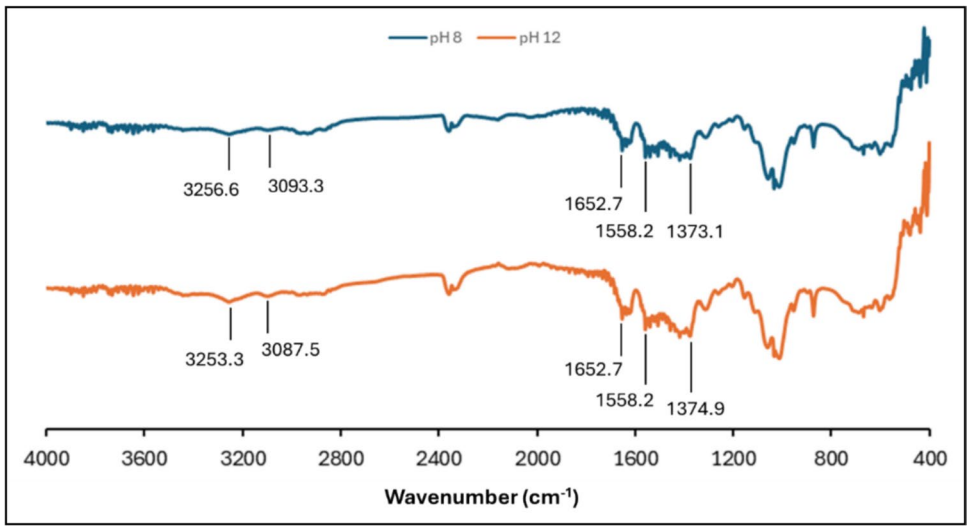


Fig. 14 FTIR spectrum of extracted shrimp shell chitin conducted at pH 8 and pH 12

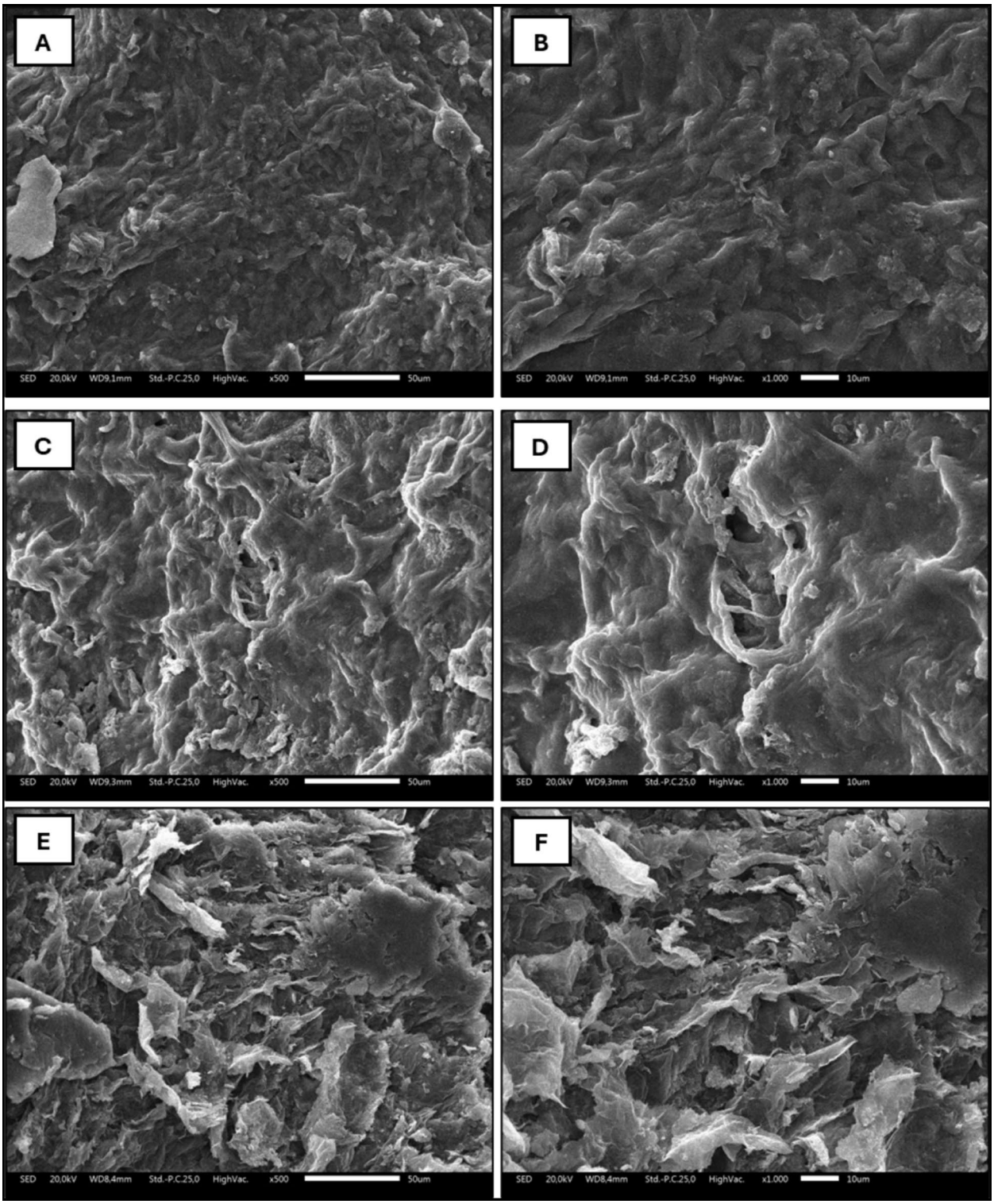


Fig. 15 SEM images of shrimp shells before adding MvCP (**A**, **B**); after treatment with MvCP at pH 8 (**C**, **D**); and after treatment with MvCP at pH 12 (**E**, **F**)

Conclusion

A novel myxobacterial strain, identified as *Myxococcus virescens* strain S2-2, was isolated from mangrove soil. This strain demonstrated the production of protease (MvCP) when cultivated in a medium containing agro-industrial byproducts such as soybean meal and tofu dregs. This strategy enhances the efficiency of bioprocess while providing an eco-friendly alternative to conventional substrates, utilizing readily available agro-industrial byproduct resources. The stability of MvCP in several industrial detergents indicates its possible usage as an additive in local household cleaning solutions. Additionally, MvCP could potentially be utilized for extracting chitin from shrimp shells, offering a valuable approach to valorizing shrimp shell waste.

Author Contribution ZKB, CR, ESE conducted the main investigation. ZKB, CR, ESE are equal as main contributors. RMS, AMD, TM are supporting investigators. ZKB and CR analyzed the data, wrote, and revised the paper. GHE, PCM, JW, and MS performed review and editing the paper.

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Data Availability The sequence data reported in this study has been deposited in the GenBank database under accession number PP789695.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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