CHARACTERIZATION OF EXTRACELLULAR PROTEASE FROM *STENOTROPHOMONAS RHIZOPHILA* MT1 ISOLATED FROM AQUACULTURE SLUDGE WASTE

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(Received 25th Nov 2021; accepted 2nd May 2022)

Abstract. A protease-producing *Stenotrophomonas rhizophila* MT1 isolated from sludge samples of shrimp ponds was selected to evaluate extracellular proteases. Enzyme activity reached the highest value of 139.02 U/mL after 60 h of culture. The isolate produced the highest protease activity in the culture medium containing 1% casein (w/v) with an inoculum size of 10% (v/v) and an agitation speed of 180 rpm. Zymography indicated two proteolytic bands with estimated molecular weights of 30 kDa and 110 kDa, respectively. The protease activity increased in Ca^{2+}, Mg^{2+}, Co^{2+} and K^{+} ions and was partially inhibited in the presence of Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} ions. Meanwhile, acetone and hexane solvents enhanced protease activity. This is the first report that evaluated the extracellular proteases produced by *S. rhizophila*. The isolate is a promising candidate for application in the removal of protein residues in aquaculture sludges, minimizing the negative effect of aquaculture sludges on the environment.
Keywords: *bioremediation, hydrolytic enzyme, organic pollutants, proteolytic activity, shrimp wastewater*

Introduction

Aquaculture is an important food production industry and has become a significant economic activity in many countries (Hamza et al., 2017; Santos and Ramos, 2018). However, a large amount of excess feed, animal carcasses and manure have been generated, exacerbating the accumulation of organic matters in the aquaculture ponds causing the blossom of harmful microorganisms as well as problem in waste sludge treatment (Li et al., 2020; Mariane De Morais et al., 2020). The major nitrogen sources of aquaculture ponds contain large amounts of protein and amino acids. Thus, sludge waste has higher organic matters, total nitrogen and phosphorus values than regular soils. Shrimp pond solid waste comprises 1.92% organic C, 0.54% total N and 1.70% P (Tangguda et al., 2015). Interestingly, the high levels of organic matters, nitrogen and phosphorus in the sediments make pond sediments a potential organic material for fertilizer use. Production of organic fertilizers from solid waste and its later use in agriculture and fisheries is recommended to reduce waste disposal volumes, environmental degradation and increase soil productivity (Suwoyo et al., 2020).

In particular, organic matters should be first decomposed into dissolved form. Then, ammonification, nitrification and denitrification possess organic matters into nitrogen gas. Bacteria play an important role in these processes by secreting enzymes such as

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_24092423

protease, nitrate reductase, nitrite reductase, nitric-oxide reductase and nitrous oxide reductase … (Su et al., 2020). Extracellular proteases produced by bacteria are important enzymes that efficiently hydrolyze organic matters into peptides and amino acids (Su et al., 2020). Protease-producing bacteria not only improve protein digestibility and host growth but also reduce organic pollutants in aquaculture (Shi et al., 2016; Amin, 2018). Protease-producing bacteria belong to four major phyla of Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Su et al., 2020).

The genus *Stenotrophomonas* belongs to the class Gamma-proteobacteria and becomes interesting due to its wide prevalence in diverse habitats with biotechnological applications (Ryan et al., 2009). Their plant growth-promoting properties and antagonistic activity against soil pathogens have been reported (Ryan et al., 2009). The genus *Stenotrophomonas* includes several species, including *S. maltophilia, S. africana, S. rhizophila, S. pavanii, S. humi, S. koreensis, S. chelatiphaga, S. dokdonesis, S. panacihumi, S. terrae, S. nitritireducens, S. acidaminiphila, S. bentonitica, S. ginsengisoli and S. daejeonensis* ... (Patil et al., 2016). Among them, *S. maltophilia* is the most studied species that produces alkaline protease and a variety of beneficial extracellular metabolites (Wang et al., 2016b). However, development for commercial use of *S. maltophilia* has been hampered by its ability to cause infections in human (Brooke, 2012) *S. rhizophila* is closely related both phylogenetically and ecologically to *S. maltophilia* (Pinski et al., 2020). However, unlike *S. maltophilia*, *S. rhizophila* has no pathogenic features for human (Berg and Martinez, 2015). *S. rhizophila* offers biotechnological applications that do not pose any risk to human health.

Although Stenotrophomonas is ubiquitous, *S. rhizophila* is commonly associated with plant rhizomes, such as maize, wheat, rice, canola, potatoes, strawberries, alfalfa, and sunflower (Ryan et al., 2009). Because of beneficial plant interactions that promote plant growth, *S. rhizophila* has become essential for agricultural biotechnology applications. On the other hand, *S. rhizophila* produces many hydrolytic enzymes such as chitinase (Jankiewicz et al., 2020), endoglucanase (Singh et al., 2015), lipase (Said et al., 2019) and protease (Steinmann et al., 2018; Singh et al., 2015). However, there is no report on the production or characterization of extracellular protease of *S. rhizophila*. Furthermore, its wide conversion properties of organic compounds combined with high metal tolerance make *S. rhizophila* is more attractive for bioremediation (Sun et al., 2021). To facilitate application of wastes into fertilizers, the selection of microbial strains that have both the properties of converting wastes into fertilizers and plant growth promotion has greater advantages value. On the other hand, the quality of fertilizers obtained from wastes depends on the extracellular enzymes including protease. Thus, the present study focused on isolating and characterizing the protease producing bacteria isolated from aquaculture sludge waste which could be interesting for converting aquaculture wastes into agriculture fertilizers.

Materials and methods

Isolation and identification of protease produced strain

The waste sludges were collected from shrimp pond in Phong Dien district, Thua Thien Hue province, Vietnam. The composition of the solid waste included 190.8 ± 4.4 mg/kg of total organic carbon, 22.6 ± 0.1 g/kg of total phosphorus and 32.0 ± 0.9 g/kg of total nitrogen. One milliliter of shrimp pond sludge waste was mixed with 9 mL of sterile distilled water, then serially diluted to 10^{-4}. One hundred

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DOI: http://dx.doi.org/10.15666/aeer/2003_24092423

microliters of the sample from the last dilution were placed onto the surface of casein agar in a petri dish (1% casein and 2% agar) and incubated at 35 °C for 72 h. The clear zone around the colonies was assessed as indication for protease activity. The colony with the greatest clearance zone on casein agar was selected and purified on LB medium.

Bacterial strains with strong protease activity were identified by molecular techniques. The bacterial DNA was extracted according to Sambrook et al. (2001). Total DNA was used as a template for PCR amplification of 16S rRNA sequences using primer pairs of 27-F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-R (5'-GGTTACCTTGTTACGACTT-3'). PCR components included 6 µL GoTaq Green master mix (Promega, USA), 10 pmol per primer, 50 ng genomic DNA and 12 µL distilled water. PCR reaction was performed on an MJ Mini^{TM} Gradient Thermal Cycler (BioRad, USA). The PCR cycles was carried out by denaturing at 95 °C for 5 min, flowing thermal cycle included steps of denaturation of 95 °C for 1 min, annealing at 55 °C for 1 min, extension of 72 °C for 1 min 30 s, repeat with 30 cycles, and final extension of 72 °C for 10 min. PCR products were qualified on 0.8% agarose gel electrophoresis. PCR products were performed nucleotide sequencing using a commercial sequencing service (Firstbase, Malaysia). Nucleotide sequences were compared with data on GenBank. Phylogenetic trees were constructed using MEGA 11 software with the Maximum Likehihood method (Tamura et al., 2021).

The growth and protease production of bacterial strain

The highest protease producing isolate was selected for further investigation. The strain was cultured in LB medium for 16 h. Then, cells suspension (1%, v/v) was transferred to protease production medium containing 1% casein, 0.2% $(NH_4)_2SO_4$; 0.1% K_2HPO_4; 0.1% $MgSO_4.7H_2O$; 0.05% NaCl, pH 7.5 and continuously cultured at 35 °C for 96 h, 180 rpm of shaking. Extracellular proteases were collected every 12 h by centrifugation at 10.000 rpm for 15 min at 4 °C. Cell growth was monitored by measuring absorbance at 600 nm (Wang et al., 2016a).

Protease activity assay

Protease activity of bacterial strains was determined by Sigma's method using casein as a substrate. One unit of protease activity was defined as the amount of casein hydrolyzing enzyme releasing an amino acid equivalent of 1 µmole of tyrosine in 1 min at pH 7.5, temperature of 37 °C (Cupp-Enyard, 2008; Marathe et al., 2018). Briefly, five milliliters of 0.65% casein substrate were incubated at 37 °C for 5 min. Then, one milliliter of crude enzyme was added and incubated for 10 min. The reaction was terminated by the adding of 5 mL of 5% trichloroacetic acid and kept for 30 min at room temperature. The mixture was then centrifuged at 6000 rpm for 10 min, 4 °C and harvested supernatant. Color was developed by adding 5 mL of 0.5 M Na_2CO_3 and 1 mL of 1 M Folin reagent into reaction supernatant, then incubated for 30 min in the dark. After incubation, the sample absorbances were measured at 660 nm using a UV-2650 Spectro UV-VIS RS Auto Spectrophotometer (Labomed, USA). Use the Tyrosine standard curve to calculate the enzyme activity according to *Equation 1:*

$$U = \frac{\mu mole\ Tyrosine * 11}{10 * 2 * 1} \quad \text{(Eq.1)}$$

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where: U is enzyme unit; 11 is the total volume (in milliliters) of assay; 10 is the time of assay (in minutes); 1 is volume of enzyme used (in milliliters); 2 is volume used (in milliliters).

Effect of culture conditions on protease production

Effect of casein concentration

The effect of substrate concentration on protease production was determined by culturing the isolate in the protease-producing medium as described above containing casein at concentrations of 0, 1, 2, or 5% (w/v). Culture was performed in 96 h at 180 rpm of shaking. Extracellular proteases were collected every 12 h by centrifugation at 10.000 rpm for 15 min at 4 °C and enzyme activity was measured according to the protease activity assay.

Effect of inoculum size

To determine the effect of inoculum size, culture was incubated in the medium with inoculum ranging from 1, 5, 10 or 15% (v/v). Reaction mixtures were incubated at 35 °C by shaking at 150 rpm for 96 h. Enzyme activity was measured every 12 h as mentioned above.

Effect of agitation rate

After determining the casein concentration and inoculum size, the effect of agitation rate on protease production was investigated. Agitation rates were investigated at 120, 150, 180 or 210 rpm for 96 h at 35 °C. Extracellular protease activity was determined for every 12 h.

Zymogram

Zymogram was performed according to Wang et al. (2016). Fifteen microliters of the cell-free supernatant were mixed with 2X loading dye buffer. The mixture is then subjected to SDS-PAGE with 5% stacking gel and 12% separating gel containing 0.8 mg/mL of casein. The gel was washed twice with Triton X-100 2.5% (v/v) for 30 min at room temperature and three times with distilled water, then incubated in the reaction buffer (50 mmol/L Tris - HCL pH 8.3, 50 mmol/L $CaCl_2$) at 35 °C for 2 h. Gel was stained with Coomassie Brilliant Blue R-250 and destained in acetate methanol solution. The appearance of a clear zone on the blue background of the gel was accessed for protease activity (Wang et al., 2016a).

Effects of pH and temperature on protease activity

The optimal pH for protease activity was determined using casein 0.65% (w/v) as substrate at different pH values. Enzyme was incubated with reaction buffers of pH ranged from 2 to 12 using difference buffers including glycine-HCl buffer (pH 2-3), sodium acetate (pH 4-5), sodium phosphate buffer (pH 6–8) and glycine-NaOH (pH 9–12)). The reactions were performed at 37 °C.

To determine the effect of temperature on protease activity, the incubation time of enzyme substrate mixture was maintained at 10 min and the temperatures for which activity of enzyme was conducted at 20 °C, 30 °C, 40 °C, 50 °C, 60 °C and 70 °C. The

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relative activities of the protease were measured. The maximum activity of crude enzyme was expressed as 100%.

Effects of inhibitors and metal ions on protease activity

The effect of metal ions on protease was examined by incubating the reaction mixture including enzyme solution, casein substrate with ion metals of Cu^{2+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and K^{+} at a concentration of 1 mM with optimum pH (pH 9) and temperature (50 °C) for 1 h. The control sample was carried out as no metal ion presence in reaction solution.

The effects of different enzyme inhibitors on protease activity were examined using ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol. The protease was pre-incubated at 25 °C for 1 h with each agent at a final concentration of 1 mM. Controls were pre-incubated without inhibitors.

Effect of organic solvents on protease activity

The protease activity was determined in the presence of 30% organic solvent (v/v) at an optimum temperature of 50 °C and pH 9. Methanol, ethanol, isopropanol, acetone and hexane were used as organic solvents in the reaction mixture. Protease activity in the solvent-free sample was used as a control.

Statistical analysis

All experiments were performed in triplicate. Absorbances were recorded three times and average values were accessed for statistical analysis of experimental replication. Data were analyzed using Statgraphics 19 software and presented as mean ± SD. The difference between means was assessed by ANOVA and Duncan's test then was used to compare data among treatments. Statistical significance between treatments was based on $p < 0.05$.

Results and discussion

Isolation and identification of protease producer

Waste sludge was collected from shrimp ponds in Phong Dien district, Thua Thien Hue province, Vietnam and delivered to the laboratory to be diluted to 10^{-4} and spread on a plate containing medium Casein agar. Three distinct bacterial colonies with the zone of clearance on casein agar were obtained after incubation, named MT1, MT2 and MT3, respectively. Among them, strain MT1 had the greatest zone of clearance on casein agar, showing that it is a good protease producer (*Fig. 1D*). The isolate was grown on protease production medium and enzyme activity was evaluated on an agar plate. *Figure 1D* showed that strain MT1 had a halo ring diameter of 25.67 ± 1.2 mm after 48 h of incubation at 35 °C (*Fig. 1D*).

The isolate MT1 was Gram negative, catalase positive, motility and rod shape. The 16S rRNA sequence of the MT1 strain was amplified, sequenced and compared with other bacterial strains available in NCBI database by BLAST analysis. The results indicated the sequence of isolate had high homology (100%) with other *S. rhizophila* species. The phylogenetic tree based on the 16S rRNA gene sequence was shown in *Figure 2*. The MT1 strain was identified as *S. rhizophila* (accession number: MZ396455).

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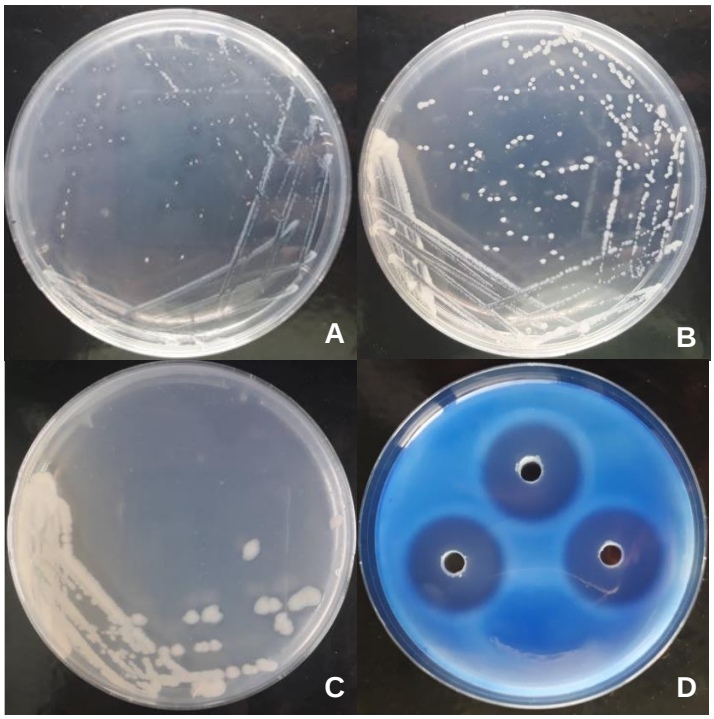

Figure 1. *Colony formation and extracellular protease activity of isolates. Colony formation of isolate MT1 (A), MT2 (B), and MT3 (C) grow on LB medium containing 1% casein. Casein hydrolytic activities are indicated as clear zones around colonies. (D) Extracellular protease activity of MT1*

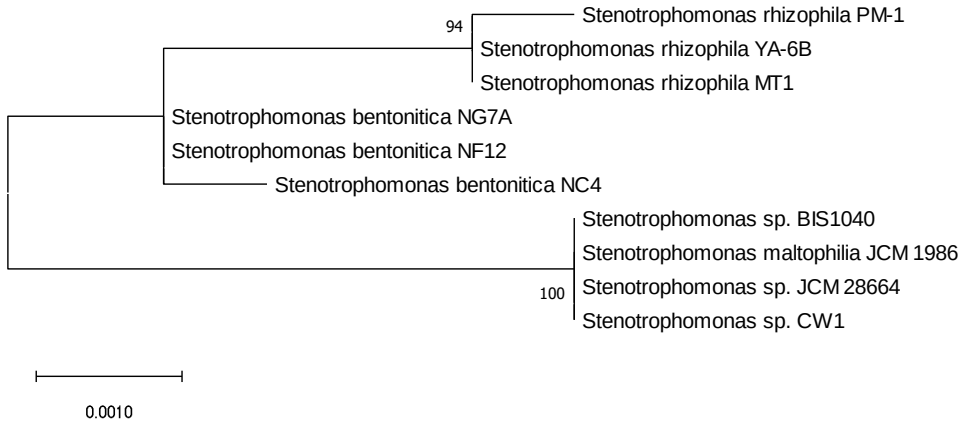

Figure 2. *Phylogenetic tree between S. rhizophila MT1 and other Stenotrophomonas species. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches*

The growth and protease production

The growth and enzyme production of *S. rhizophila* MT1 were examined on medium supplemented with 1% casein (w/v) for 96 h at 35 °C. The results shown in *Figure 3* indicated proteolytic activity was hardly detectable in the early stages of culture. Meanwhile, protease production started only up-regulated at the log phase to the end of

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the growth curve. The proteolytic activity reached the highest activity between 48 h to 60 h culture (no significant difference) with a peak at 60 h (139.02 U/mL) and then decreased. The production of protease was observed to be proportional to the growth of the organism. The results are consistent with the study by Wang et al. (2016) whereas protease production of *S. maltophilia* FF11 is proportional to the growth and reaches maximal activity at the peak of the growth (Wang et al., 2016a).

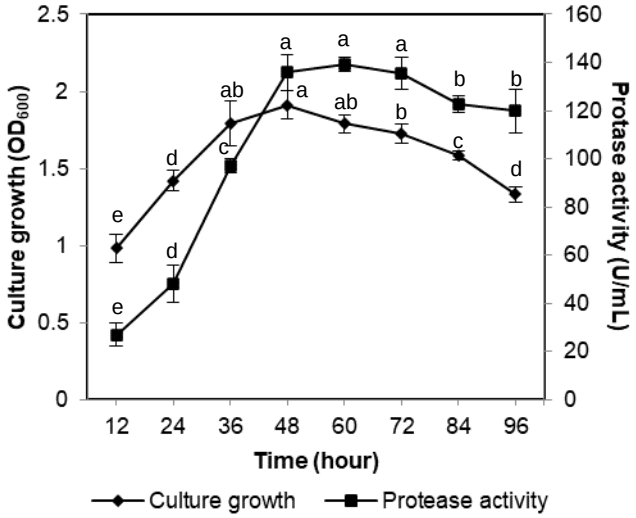

Figure 3. *The growth and extracellular protease activity by S. rhizophila MT1. Error bars represent the standard deviations of the mean (n = 3). Different letters indicate significant differences ($p < 0.05$)*

Effect of culture conditions on protease production

Effect of casein concentration

The concentration of casein was investigated by conducting experiments with increasing the concentration of casein in the medium. *Figure 4A* showed that protease achieved the highest activity of 139.02 ± 2.87 U/mL in the medium supplemented with 1% casein. When the casein concentration increased to 2 and 5%, the enzyme activity dramatically decreased to 135.16 ± 5.31 and 76.21 ± 3.79 U/mL, respectively. Casein has been reported as major nitrogen source for many microorganisms for maximal protease production. Jayasree et al. (2009) reported 1% casein as the major nitrogen source for the alkaline protease produced by *Streptomyces pulveraceus* (Jayasree et al., 2009). *Streptomyces halstedii* Salh-12 and *Streptomyces endus* Salh40, the two most proteolytic thermophilic strains used 1% casein as major nitrogen source for protease production, respectively (El Zawahry et al., 2007). Meanwhile, Asha et al. (2018) reported *Bacillus cereus* FT1 secreted the highest enzyme activity under cultivation in medium supplemented with 3.5% casein concentration (Asha and Palaniswamy, 2018).

Effect of inoculum size

The finite volume of the culture medium means that it contains only limited nutrients for microorganisms. The consumption of nutrients gradually depends on the bacterial population. Inoculum size is important in optimizing production because very low

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initial biomass concentrations may lead to long incubation times, while high inoculum levels lead to rapid biomass gain, resulting in nutritional stress affecting product formation (Khursade et al., 2019). Therefore, to ensure the production of enzymes in a limited media, it is necessary to control the initial stocking bacterial population. The maximal protease activity achieved at 188.56 ± 1.77 U/mL with inoculum size of 10% (v/v). The results showed that the *S. rhizophilia* MT1 required a higher initial inoculum size than *Bacillus licheniformis* NK reported by Ramkumar et al. (2018) and *B. cereus* AT reported by Vijayaraghavan et al. (2014) with the inoculum size of 5 and 6% (v/v), respectively (Ramkumar et al., 2018; Vijayaraghavan et al., 2014). A higher inoculum size of 15% (v/v) reduced protease production by *S. rhizophilia* MT1 than that inoculum size of 1% (v/v) (*Fig. 4B*). Therefore, high inoculum sizes do not enhance protease yield. The increase in the production of protease using small inoculum sizes is due to the higher surface area to volume ratio, which results in increasing protease production (Rahman et al., 2005). In addition, the improved distribution of dissolved oxygen and more efficient nutrient uptake also contributes to higher protease production. On the contrary, if the inoculum sizes are too small, insufficient bacterial population will reduce in the amount of protease secreted (Shafee et al., 2005).

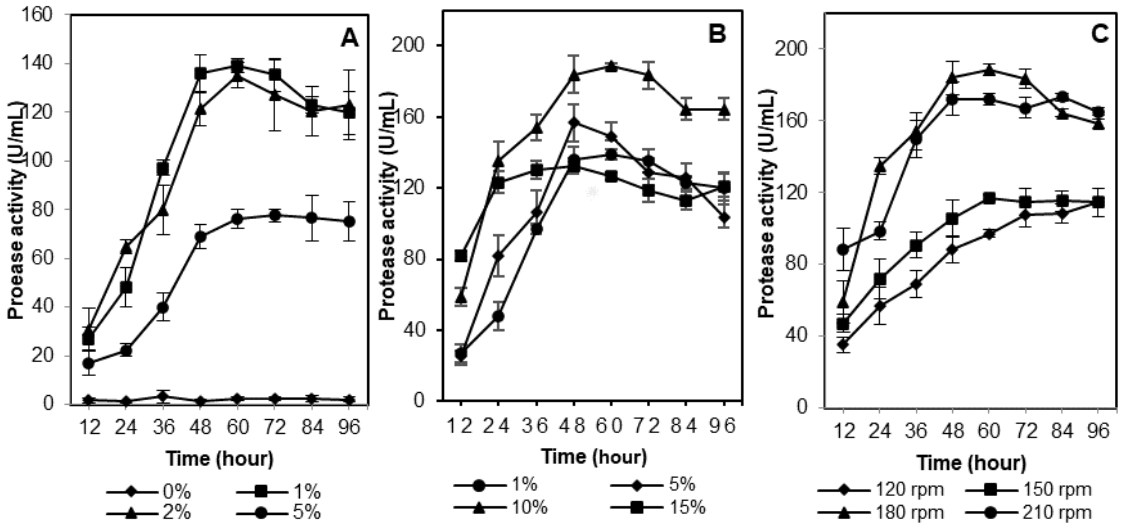

Figure 4. *Effect of casein concentration (A), inoculum size (B) agitation rate (C) on extracellular protease production by S. rhizophila MT1. Error bars represent the standard deviations of the mean (n = 3)*

Effect of agitation rate

Microorganisms differ in their oxygen requirements. In particular, oxygen acts as a terminal electron acceptor for oxidation reactions to provide energy for cellular activities. Variation in agitation speed was found to affect the degree of mixing in a shaker or bioreactor (Nascimento and Martins, 2004). The results showed that protease increases with increasing shaking speed and reached its highest at 180 rpm (188.56 ± 1.77 U/mL) (*Fig. 4C*). At this rate, aeration of the culture medium is increased, leading to an adequate supply of dissolved oxygen in the medium. Although protease production was found to decrease with shaking at 210 rpm, low shaking conditions most likely restricted protease production. Therefore, a higher shaking speed

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might increase the oxygen pressure of the system but not the yield, possibly because at high shaking speed, the enzyme's structure may not be stable (Abusham et al., 2009). Reducing the aeration rate significantly decreased protease production, showing reducing oxygen supply is an important limiting factor for growth and protease synthesis (Nascimento and Martins, 2004).

Effects of pH and temperature on protease activity

pH and temperature are factors that significantly change enzyme activity due to the abilities on modifying the catalytic groups in enzyme active sites as well as enzyme structure. Thus, each enzyme only catalyzes at a suitable temperature and pH range, where the reaction rate occurs fastest (Harris and Turner, 2002; Arcus and Mulholland, 2020). The effect of pH on protease activity was investigated with a pH range of 2.0–12.0. The protease was active with a board pH range of 7.0–10.0. The optimal pH was 9.0 (*Fig. 5A*). When pH increased from 5.0 to 8.0, the protease activity gradually increased with the relative activity increasing from 25.32 ± 3.44 to $96.82 \pm 6.02\%$, respectively. In the alkaline conditions (pH 10.0–12.0), the relative activity gradually decreased from 77.76 ± 9.43 to $27.36 \pm 2.1\%$ compared to the protease activity at pH 9.0. Protease lost $\geq 90\%$ activity at an acidic pH of 2.0-4.0. These results are in accordance with previous reports on alkaline proteases from *S. maltophilia* JSHY3 (Wang et al., 2016b), *S. maltophilia* FF11 (Wang et al., 2016a), *B. firmus* Tap5 (Joshi, 2010) and *Beauveria* sp. MTCC 5184 (Shankar et al., 2011).

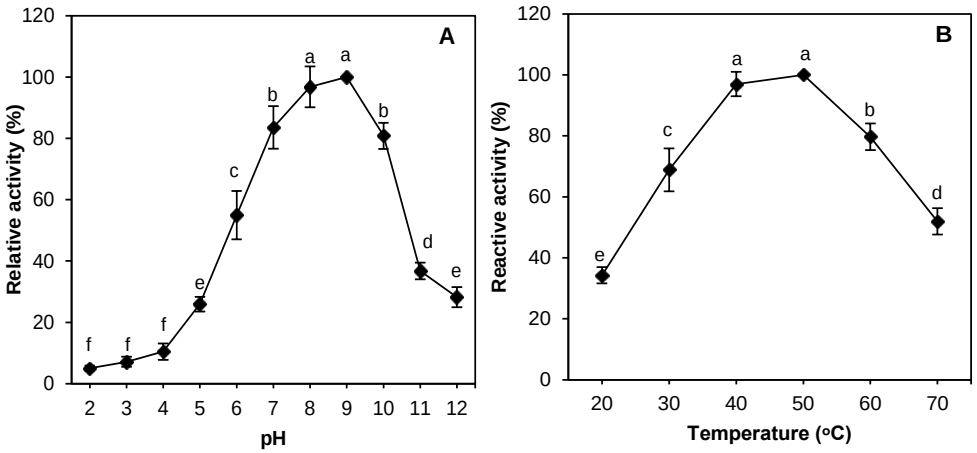

Figure 5. *Effect of pH (A) and temperature (B) on extracellular protease activity produced by S. rhizophila MT1. Error bars represent the standard deviations of the mean (n = 3). Different letters indicate significant differences ($p < 0.05$)*

The enzyme is active over a wide temperature range from 20 °C to 70 °C. A linear increase in protease activity was observed with increasing temperature. Maximum enzyme activity reached at 50 °C and maintained $\geq 50\%$ activity at temperature ranges of 30–70 °C. The lowest activity occurred when enzyme reaction was conducted at a temperature of 20 °C, reaching $34.31 \pm 2.67\%$. The relative protease activity of $51.94 \pm 4.3\%$ remained at 70 °C (*Fig. 5B*). The results are similar to previous study conducted on alkaline proteases from *Serratia marcescens subsp. sakuensis* TKU019 with maximum activity at 50 °C (Liang et al., 2010). Shankar et al. 2011 reported an

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optimum temperature of 50 °C for protease derived from *Beauveria* sp. MTCC 5184 (Shankar et al., 2011). Similar result was reported on protease from *S. maltophilia* S-1 with an optimum temperature of 50 °C (Miyaji et al., 2005).

Effects of inhibitors and metal ions on protease activity

Various metal ions have been reported to affect protease activity. Metal ions play an important role in enhancing thermal stability and maintaining the active conformation of enzymes, and influencing enzyme activity by binding at the catalytic site (Mechri et al., 2017). Calcium ion is an inducer and stabilizer of many enzymes and protecting them from structural changes (Sharma et al., 2017). The influence of various metal ions on the enzyme is presented in *Figure 6*A. The protease activity was not significantly ($p > 0.05$) affected by 1 mM of Zn^{2+}, Mg^{2+} and Cu^{2+} while the activity of enzyme was decreased in the presence of Mn^{2+}, Fe^{2+}. Meanwhile, Co^{2+}, K^{+} increased enzyme activity of 107.7%, and 108.37%, respectively. However, these increases were not significant in compared with control. On the contrary, Ca^{2+} significantly enhanced protease activity up to 116.51%. The results are equivalent to study Wang et al. (2016). However, protease of *S. rhizophilia* MT1 retained ≥ 90% activity in the presence of metal ions such as Zn^{2+}, Mn^{2+}, Cu^{2+} and Fe^{2+}, while Mn^{2+} and Fe^{2+} ions decreased protease activity of *S. maltophilia* FF11. Zn^{2+} and Cu^{2+} inhibited protease activity of *S. maltophilia* FF11 (Wang et al., 2016a). A serine protease from *B. safensis* is activated by Ca^{2+}, Co^{2+} and Mg^{2+} and inhibited by Ni^{2+} and Hg^{2+} ions (Rekik et al., 2019). Alkaline proteases from *Aeribacillus pallidus* are enhanced by Ca^{2+}, Cu^{2+} and Fe^{2+} ions (Mechri et al., 2017).

Proteins can be classified based on their susceptibility to inhibitors. The relative protease activity increased to 104.48 ± 3.91% in the presence of EDTA, a well-known inhibitor of metalloprotease and unaffected by β-mercaptoethanol (98.69 ± 4.2%), a cysteine protease inhibitor. The protease of *S. rhizophilia* MT1 expresses optimal activity at pH 9, suggesting it belongs to the alkaline protease class.

Effect of organic solvents on protease activity

The organic solvent stability of an enzyme depends on its nature (Matkawala et al., 2019). The effect of different organic solvents on protease activity was investigated at 30% (v/v) concentration. *Figure 6B* showed that the protease was not affected significantly ($p > 0.05$) by hydrophilic solvents such as methanol, ethanol and isopropanol. The enzyme activity was significantly increased to 114.81 ± 6.42 and 121.56 ± 11.6% in the presence of acetone and hexane, respectively. The effect of organic solvents on protease activity varies with protease types and solvents. It is known that proteases are more active and stable in hydrophobic solvents than in hydrophilic solvents (Wang et al., 2016a). For example, protease activities of *B. pumilus* 115b and 146 increase in hydrophobic solvents such as hexane, 1-decanol, isooctane, and n-dodecane (25%, v/v) (Rahman et al., 2005). The organic solvent stability of *S. rhizophila* MT1 protease is similar to that of protease from *S. maltophilia* FF11 (Wang et al., 2016a) and higher than other alkaline proteases produced by *Aeribacillus pallidus* C10 (Yildirim et al., 2017), *Neocosmospora* sp. N1 (Matkawala et al., 2019). In the presence of organic solvents, the ability of natural proteases to remain stable without making any modifications to enzyme stability is important for various applications (Doukyu and Ogino, 2010). The strong stability of *S. rhizophilia* MT1 protease in organic solvents leads to a great applicability advantage.

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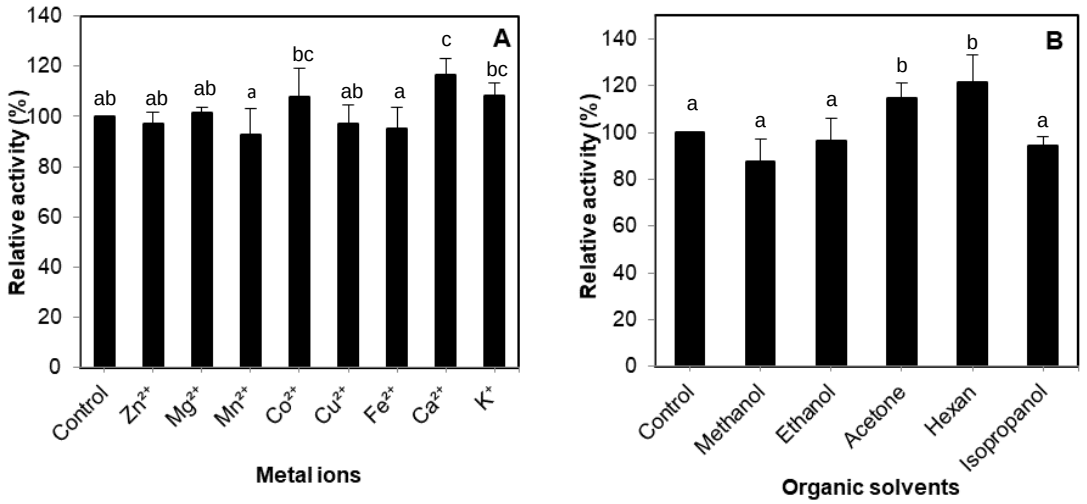

Figure 6. *Effect of metal ions (A) and organic solvents (B) on extracellular protease activity produced by S. rhizophila MT1. Error bars represent the standard deviations of the mean (n = 3). Different letters indicate significant differences (p < 0.05)*

Zymogram

Molecular mass and enzyme activity were assessed using zymogram electrophoresis. The cell-free supernatant of *S. rhizophila* MT1 was subjected to polyacrylamide gel electrophoresis to separate and determine the molecular weight of the extracellular proteases. The zymogram showed two distinct casein-resolution luminous areas on the gel with molecular masses of 30 and 110 kDa (*Fig. 7*). Previous studies reported that alkaline proteases from *S. maltophilia* G2 (Huang et al., 2009), *B. licheniformis* MP1 (Jellouli et al., 2011), *Beauveria* sp. MTCC 5184 (Shankar et al., 2011), *B. halodurans* JB 99 (Shrinivas and Naik, 2011) exhibit similar small protease band of *S. rhizophila* MT1. Meanwhile, alkaline proteases of *P. fluorescens* (Kandasamy et al., 2012); *S. maltophilia* FF11 (Wang et al., 2016a) are differed. Numerous higher molecular weight of alkaline proteases have been reported from *B. halotolerans* CT2 (Dorra et al., 2018) and *Aspergillus fumigatus* TKU003 (Wang et al., 2005).

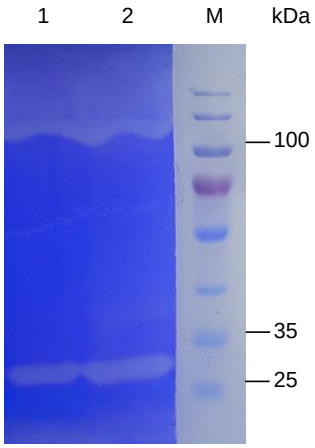

Figure 7. *Proteolytic zymogram of S. rhizophila MT1 cell-free supernatant. Lane M: PageRuler™ Prestained Protein (Thermo Scientific, USA); Lane 1, 2: S. rhizophila MT1 cell-free supernatant*

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Conclusion

The *S. rhizophila* MT1 isolate shows strong protease production. The maximum enzyme activity occurs in the culture medium containing 1% casein (w/v), inoculum size of 10% (v/) and agitation rate of 180 rpm. Two clear caseinolytic activity protein bands had molecular weights of 30 kDa and 110 kDa. The optimum activity of this enzyme expressed at temperature of 50 °C and pH of 9. Ca^{2+}, Co^{2+} and K^{+} ions greatly enhanced enzyme activity. The enzyme was stable in the presence of the metal ions Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} with protease activity maintained $\geq$ 90% maximal activity. Organic solvents such as acetone and hexane solvents increased enzyme activity. The present study demonstrates that *S. rhizophila* MT1 protease is stable in the environment containing various metal ions and organic solvents, promising the potential to convert aquaculture sludge to agriculture fertilizers, which minimizes the negative effect of aquaculture sludge waste to environment.

Acknowledgements. The study received funding support from Vietnam Ministry of Education and Training under grant number B2020-DHH-18.

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