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Screening of proteolytic, amylolytic, and lipolytic bacteria and their consortia for concomitant production of hydrolytic enzymes and bioremediation of shrimp pond sludge ☆

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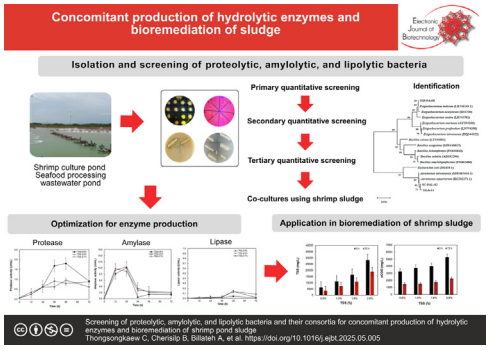
GRAPHICAL ABSTRACT

Screening of proteolytic, amylolytic, and lipolytic bacteria and their consortia for concomitant production of hydrolytic enzymes and bioremediation of shrimp pond sludge.



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ABSTRACT

Background: Aquaculture has become the fastest-growing sector in recent decades, and this has led to intensified cultural practices to achieve high yields. However, such practices have raised concerns regarding their environmental impact, as aquaculture sludge and wastewater cause significant organic pollution. This study then aimed to isolate and screen proteolytic, amylolytic, and lipolytic bacteria and evaluate their consortia for enzyme production and bioremediation of shrimp pond sludge.
Results: The strategy using multiple substrates in the isolation media successfully obtained bacterial strains with multiple hydrolytic activities. After primary and secondary quantitative screening, 18 isolates that exhibited high dual and triple hydrolytic activities were selected. After tertiary quantitative screening using synthetic shrimp pond sludge and co-culture tests, *Exiguobacterium indicum* SSP-PA-08, *Bacillus coagulans*, and *Bacillus subtilis* were selected due to their synergy for the production of triple hydrolytic enzymes. Their consortia inoculated in shrimp pond sludge containing 0.8% total

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Pond sludge
Proteolytic bacteria
Shrimp feed residues
Synergistic interaction

suspended solids (TSSs) showed an increase in proteolytic activity by 2.5 folds and amylolytic activity by 20 folds, which led to a greater reduction in TSS. The highest enzyme production was obtained using shrimp pond sludge containing 1.6% TSS.
Conclusions: This study has developed the methods to isolate bacteria with multienzyme-producing ability. Co-culturing these bacteria in synthetic shrimp sludge significantly enhanced hydrolytic activity and led to a greater reduction in TSS. These strategies may contribute greatly to the hydrolytic enzyme production and environmentally friendly bioremediation of aquaculture sludge.
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1. Introduction

Aquaculture has become the fastest-growing sector in recent decades, with an average annual growth rate exceeding 8% [1]. This rapid expansion has led to intensified cultural practices to achieve high yields. However, such practices have raised concerns regarding their environmental impact, as aquaculture sludge and wastewater cause significant organic pollution. Aquaculture sludge, such as sludge from shrimp ponds, accumulates at the bottom of ponds and requires regular removal due to its high accumulation rates. It mainly contains nitrogenous compounds, phosphorus, and other dissolved organic carbon. Sludge can impact habitat availability for cultured animals and generate toxic substances that threaten aquatic life. Due to these harmful effects, sludge must be removed from culture ponds regularly and treated appropriately before being discharged into receiving water bodies [1,2].

Shrimp pond sludge mainly forms as a result of excessive feed, animal waste, mineral sediments, phytoplankton, airborne debris, decaying plant material, and residues from therapeutic and prophylactic treatments [2]. Common concerns about water quality in shrimp aquaculture include the content of total suspended solids (TSSs), nitrogen, chemical oxygen demand (COD), biochemical oxygen demand (BOD), and dissolved oxygen (DO) [3]. Treating shrimp pond sludge is crucial for ensuring a healthy ecosystem and enhancing the sustainability of aquaculture practices [4]. The traditional way to treat the sludge released from aquaculture systems is by drying and adding lime. These methods may cause problems resulting in the incomplete degradation of organic matter. Recently, there has been growing attention to applying promising bacteria and enzymes in a variety of industries and also bioremediation in aquaculture systems [5,6,7,8]. Theoretically, beneficial bacteria hold significant promise for degrading sludge. While most researches focus on bioremediation of wastewater and aquaculture water, the bioremediation of solid waste, such as sludge, remains underexplored.

This study aimed to bioremediate shrimp pond sludge using multienzyme-producing bacterial consortia. Given that shrimp pond sludge is primarily composed of organic compounds including proteins, carbohydrates, and lipids, the bacteria should produce a range of enzymes to hydrolyze these compounds into water-soluble forms and efficiently bioremediate them. This approach is expected to biologically treat shrimp pond sludge and mitigate toxicity from sludge accumulation. Utilizing proteolytic, lipolytic, and amylolytic bacterial consortia would present an efficient and environmentally sustainable alternative, potentially reducing the need for harmful chemicals to treat the sludge.

2. Methods and materials

2.1. Sample collection

Soil and water samples were taken from the shrimp culture pond after the harvesting period and the seafood processing plant in Songkhla, Thailand. All samples were collected in sterilized plastic bottles and bags and then immediately transported to the laboratory and stored at 4°C until use. Bacterial strains with high hydrolytic activities from the culture collection of the Microbiological Laboratory, Faculty of Agro-Industry, Prince of Songkla University were also used in this study. These include *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus* sp. B.24, *Bacillus* sp. LN008, and *Bacillus* sp. MK007.

2.2. Isolation and primary screening of multienzyme-producing bacteria

2.2.1. Isolation using selective agar media

The collected water and soil samples were enriched in synthetic shrimp pond wastewater, prepared by using a suspension of shrimp feed (Manee samute farm, Songkhla, Thailand) at 1.2% w/v and adjusted pH to 7.0. The shrimp feed is composed of 42% proteins, 37% carbohydrates, and 6% lipids. For isolation and primary screening of protease, lipase, and amylase-producing bacteria, the enriched samples were serially diluted with normal saline (0.85% sodium chloride). Each dilution was spread on selective agar medium for isolation of protease and amylase producing bacteria (PA medium), which was composed of (g/L): skimmed milk 100, soluble starch 2, peptone 10, calcium chloride 0.1, agar 15, distilled water 1000 mL, pH 7.0 [9]; selective medium for isolation of lipase and amylase producing bacteria (LA medium), which was composed of (g/L): Tween 80 10, soluble starch 2, peptone 10, calcium chloride 0.1, agar 15, distilled water 1000 mL, pH 7.0 modified from Gozari et al. [10]; and selective medium for isolation of protease, amylase and lipase producing bacteria (PAL medium), which had two layers: one layer was composed of (g/L): skimmed milk 100, soluble starch 2, peptone 10, calcium chloride 0.1, agar 15, distilled water 1000 mL, pH 7.0 and one another layer was composed of (g/L): Tween 80 10, soluble starch 2, peptone 10, calcium chloride 0.1, agar 15, distilled water 1000 mL, pH 7.0. The cultures were incubated at 30 ± 2°C for 48 h. The bacterial colonies that showed clear zones on PA and PAL media, indicating their ability to produce protease, were selected. The bacterial colonies that showed blue color change upon the addition of iodine solution on PA, LA, and PAL media were selected as amylase-producing bacteria. Bacterial strains that showed a

clear zone on LA and PAL media were selected as lipase producers. The lipase-producing ability was also confirmed on rhodamine B-containing media [11]. All selected bacterial colonies were subcultured to verify purity and then stored at 4°C. The hydrolytic (digestion) index was calculated as shown in [**Equation 1**].

$$\text{Hydrolytic (digestion) index} = \frac{\text{Diameter of the clear zone}}{\text{Diameter of the colony}} \quad (1)$$

2.2.2. Primary quantitative screening using selective agar media

All isolates with comparatively high hydrolytic activities were selected and confirmed for their individual hydrolytic activity using corresponding selective agar media. Bacteria capable of producing protease were cultured on solid media containing skim milk powder and the clear zone around colonies indicating their proteolytic activity. Bacteria capable of producing amylase were initially grown on solid media containing soluble starch. The amylolytic activity was indicated by a blue color change upon the addition of iodine solution after bacterial colonies were formed. Bacteria capable of producing lipase were initially grown on solid media containing Tween 80 and further confirmed on rhodamine B solid medium. The hydrolytic index was then calculated as previously described.

2.2.3. Secondary and tertiary quantitative screening

In secondary quantitative screening, all isolates obtained from primary qualitative screening were quantitatively tested for their abilities to produce extracellular enzymes using a liquid medium. Each enzyme was tested in 50 mL of selective media (as mentioned in the primary qualitative screening section, excluding agar). The liquid medium was 10% v/v inoculated and incubated at 30 ± 2°C for 48 h on an orbital shaker at 150 rpm. Protease, amylase, and lipase activities were quantitatively analyzed as mentioned in the analytical section. The bacteria with the highest individual enzyme activity as well as those that showed multiple enzyme activities were selected.

In tertiary quantitative screening, the selected bacteria were cultured in synthetic shrimp pond sludge at 30 ± 2°C and 150 rpm for 48 h. The culture broth was sampled every 12 h and analyzed for protease, amylase, and lipase activities, as well as TSS removal efficiency. The bacterial strains were selected based on their high protease-producing ability (Bacteria P), high amylase-producing ability (Bacteria A), and high lipase-producing ability (Bacteria L).

2.3. Identification of bacterial strains based on 16S rRNA sequences

The 16S rRNA genes of the selected isolates were amplified using primer pairs 27F (5′ AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [12]. The PCR products were analyzed using a commercial DNA sequence analysis service (Gibthai Co. Ltd., Bangkok, Thailand). BioEdit software v.2.7.5 was used to trim poor-quality ends, and the sequences were then matched with other 16S rRNA gene sequences accessible in GenBank using the BLASTN program.

2.4. Enzyme production using synthetic shrimp pond sludge

The effects of mono- and co-cultures on enzyme production were investigated. Bacteria were grown in synthetic shrimp pond sludge with 10% inoculation. The cultures were incubated at 30 ± 2°C and 150 rpm for 72 h. The samples were taken every 12 h and analyzed for pH, TSS, soluble protein, and COD. After centrifuging at 10,000 rpm for 10 min, the supernatant was analyzed for enzyme activity. The cultures that exhibited high enzyme production and high ability to reduce TSS in sludge effluent were selected. The effect of synthetic sludge concentration on enzyme production was studied by varying sludge concentration with TSS at 0.8, 2.0, and 2.7%.

2.5. Optimization of enzyme production using shrimp pond sludge

The shrimp pond sludge was also used for enzyme production. The effect of sludge concentration was studied. The experiment was conducted in 250-mL Erlenmeyer flasks containing sterile shrimp pond sludge from a shrimp pond with TSS in the range of 0.6–2.8%. The cultures were incubated at 30 ± 2°C and 150 rpm for 48 h for 7 d. The culture broth was taken every 12 h and analyzed for pH, TSS, soluble protein, and COD. After centrifuging at 10,000 rpm for 10 min, the supernatant was analyzed for enzyme activity.

2.6. Analytical methods

2.6.1. Protease activity

The proteolytic activity was analyzed following the modified method of Ruangwicha et al. [13] with some modifications. A total of 130 μL of 1% (w/v) casein in 0.01 M phosphate buffer, pH 7, was added with 25 μL of diluted enzyme solution. The hydrolysis reaction was carried out at 37°C for 10 min, and 130 μL of 0.11 M trichloroacetic acid (TCA) was added to stop the reaction. The mixture was left for 30 min and subjected to centrifugation at 10,000 rpm for 10 min. A total of 250 μL of supernatant was added with 625 μL of sodium carbonate (500 mM) and 125 μL of Folin's reagent. The mixture was kept at 37°C for 30 min before the absorbance was measured at 660 nm using UV–VIS spectrophotometer (U-2000, Technical Cooperation, USA). One unit of protease activity was defined as the amount of enzyme required to liberate one μmole of tyrosine per min under the examined conditions.

2.6.2. Amylase activity

Amylase activity was measured using the DNSA method with some modifications [14]. In addition, 500 μL of 1% w/v soluble starch dissolved in 0.05 M phosphate buffer (pH 7) was added with 500 μL of diluted enzyme solution. The hydrolysis reaction was carried out at 50°C for 10 min. To stop the reaction, the reaction mixture was boiled for 10 min. After adding 300 μL of 3, 5-dinitrosalicylic acid reagent, the mixture was boiled for 10 min. 1.6 mL of distilled water was added after cooling down, and the absorbance was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme required to liberate one μmole of D-glucose equivalent per min under the examined conditions.

2.6.3. Lipase activity

Lipase activity was measured according to Louhasakul and Cheirsilp [15] with some modifications. The enzyme solution was added to phosphate buffer (pH 7.0, 50 mM) and 10% w/v palm oil in *iso*-octane. The hydrolysis reaction was carried out at 30 ± 2°C for 30 min using a high-speed vortex at 10,000 rpm. 0.2 mL of 6 M HCl was added to stop the reaction, and the upper phase was instantly taken. Before measurement, the upper phase was suitably diluted with *iso*-octane and added to 0.4 mL of cupric acetate-pyridine reagent. This mixture was immediately mixed for 15 s, and then, the absorbance of upper layer was measured at 715 nm. Using a standard curve of palmitic acid, the absorbance values were used to calculate the amount of free fatty acid released. One unit of lipase activity was defined as the amount of enzyme required to liberate one μmole of free fatty acids in the form of palmitic acid per min under the examined conditions.

2.6.4. Soluble protein content

After centrifugation at 10,000 rpm for 10 min, the supernatant of the samples was analyzed for soluble protein content by the Lowry protein assay [16]. The properly diluted 0.2 mL of sample solution was added with 2.1 mL of solution A (consisting of 1% $CuSO_4$ 5 H_2O, 1% sodium potassium tetrahydrate, and 2% Na_2CO_3 in 0.1 N NaOH at a ratio of 1:1:98). The reaction mixture was incubated at 30 ± 2°C for 10 min before added with 0.2 mL of solution B (Folin-Ciocalteu reagent added with water at a ratio of 1:1) and incubated for 30 more min. The optical density was determined using a spectrophotometer set to a wavelength of 660 nm. The recorded absorbance values were then compared to a standard curve for Bovine Serum Albumin (BSA).

2.6.5. Total suspended solid (TSS)

The filter paper was placed in an oven and dried at a temperature range of 103–105°C for 1 h. Subsequently, the dried filter paper was weighed after cooling down. The filter paper was placed in a Buckner filter funnel that was connected to a vacuum pump. Then, the sample of 10 mL was poured onto filter paper and turned on the vacuum pump until the water had evaporated. The filter paper with the sample was dried in an oven at 105°C for 1 h. TSS values were then calculated from the difference between the filter paper weight and the filter paper with sample weight.

2.6.6. Chemical oxygen demand (COD)

The chemical oxygen demand (COD) was measured according to the standard APHA method [17]. In total, 5 mL of the sample was added to a 20 × 150 mm COD digestion tube (pre-washed with dilute 20% of H_2SO_4). After slowly adding 3 mL of 0.1 N potassium dichromate, 7 mL of sulfuric acid reagent containing $AgSO_4$ was added through the sides of the tubes. The digestion tube was transferred to the pre-heated COD block digester and heated at 150°C for 2 h. The blank was also performed by using distilled water instead of the sample. Also, 1–2 drops of Ferroin were added as an indicator. The solution was titrated against 0.1 M ferrous ammonium sulfate solution (FAS) [17].

2.6.7. Statistical analysis

The experiments were carried out in triplicates. The experimental data are expressed as means and standard deviations. One-Way Analysis of Variance (ANOVA) was used to analyze significant differences between treatments; it operates under the assumption of a null hypothesis stating no relationship or difference between the groups.

3. Results and discussion

3.1. Isolation and primary screening of multienzyme-producing bacteria

3.1.1. Isolation using selective agar media

This study aimed to isolate potential proteolytic, amylolytic, and lipolytic bacteria from shrimp ponds after the harvesting period and seafood processing wastewater ponds. The shrimp pond samples were divided into two sources: water from the shrimp pond (WSP) and sludge from the shrimp pond (SSP). The samples from seafood processing plant were also divided into two sources: sample from scum pond (SC) and sample from aerobic hydrolytic pond (HP). The samples from each source were enriched in synthetic shrimp pond wastewater at 30 ± 2°C with a shaking speed of 150 rpm for 48 h before isolation of bacteria using selective agar media. The medium used for simultaneous isolation of proteolytic and amylolytic bacteria was defined as PA medium, and that used for isolation of lipolytic and amylolytic bacteria was defined as LA medium. The medium used for isolation of bacteria with the ability to concomitantly produce triple hydrolytic enzymes (protease, amylase, and lipase) was defined as PAL medium. The bacterial colonies that showed clear zones on PA and PAL media, indicating their ability to produce protease, were selected. The bacterial colonies that showed blue color change upon the addition of iodine solution on PA, LA, and PAL media were selected as amylase-producing bacteria. Bacterial strains that showed a clear zone on the Tween 80 layer of LA and PAL media were selected as lipase producers. Based on the clear zone developed on agar media, a total of 123 isolates were obtained from shrimp ponds, while only 41 isolates were obtained from seafood processing plants (**Table S1**). This difference might be attributed to the different compositions of the sample sources. It has been reported that the carbon and nitrogen ratio level influences microbial community and their growth [18]. It should be noted that more bacteria were detected in the samples from wastewater than in that from the sludge, likely because the water sample contains more soluble nutrients and provides better mass transfer than the sludge.

3.1.2. Primary quantitative screening using selective agar media

All isolates with high hydrolytic activities were selected and confirmed for their individual hydrolytic activity using the corresponding selective agar media. The digestion index, namely the ratio of the diameter of the clear zone to the diameter of the colony, was measured. Among the strains screened, 18 isolates that exhibited comparatively high proteolytic, amylolytic, and lipolytic activities were selected. Table 1 shows that the use of multiple substrates in the isolation media can isolate bacterial strains with triple hydrolytic activities. Notably, the isolates obtained from shrimp pond samples demonstrated higher proteolytic, amylolytic, and lipolytic activities than those obtained from seafood processing wastewater pond samples. This was likely due to the difference in the availability of the nutrients in the sample sources, as the sludge samples might contain more complex nutrients. Among the strains selected, isolate WSP-PA-06 exhibited the highest proteolytic activity index (PI = 5.5), followed by SC-PA-03 (PI = 4.33) and HP-PA-01 (PI = 4.0) in the primary screening. Interestingly, these strains also showed high amylolytic activity and lipolytic activity index while isolate SSP-PA-08 and WSP-PA-01 exhibited the highest amylolytic activity index (AI = 5.0) and the highest lipolytic activity index (LI = 4.0), respectively. These results have confirmed that it was possible to isolate bacterial strains with multiple hydrolytic activities using the developed strategy. Until now, only a few studies have developed methods to isolate bacteria with multiple hydrolytic activities. Shaik et al. [9] reported that the bacteria, when cultivated in liquid medium containing mixed substrates of casein, starch, and tributyrin, were capable of producing triple hydrolytic activities of protease, amylase, and lipase. Al-Dhabi et al. [19] reported that when using a medium containing 1% olive oil and casein as substrates, the isolates exhibited both protease and lipase activities. However, there is no extensive study on their concomitant production of hydrolytic enzymes and their application in the bioremediation of sludge.

3.2. Secondary and tertiary quantitative screening

3.2.1. Secondary quantitative screening on selective liquid media

As the primary screening of enzyme-producing bacteria on solid medium and measuring enzyme activity using the digestion index have limitations in terms of mass transfer and nutrient availability compared to cultivation in liquid media, the secondary quantitative screening was performed by culturing in a selective liquid medium to quantitatively measure the enzyme activity. The selected 18 isolates from primary screening were screened and compared with the bacterial strains with high hydrolytic activities in the culture collection at Microbiology Laboratory, Faculty of

Table 1
Hydrolytic activity of eighteen isolates from shrimp pond and seafood processing plant during isolation and primary screening.

Sources	Isolate codes	Index during isolation		Index in primary screening			
		PI	AI	LI	PI	AI	LI
Water in shrimp pond (WSP)	WSP-PA-01	3.0	2.0	–	3.0	2.0	4.0
	WSP-PA-02	3.9	1.4	–	4.0	1.24	3.2
	WSP-PA-06	5.5	3.6	–	5.5	3.5	2.0
	WSP-LA-02	–	2.9	1.2	1.88	3.0	1.2
	WSP-LA-03	–	1.8	2.3	1.36	1.7	2.5
	WSP-LA-04	–	3.4	1.4	2.0	3.4	1.4
	WSP-LA-08	–	2.2	1.5	1.71	2.2	1.5
Sludge in shrimp pond (SSP)	SSP-PA-05	3.0	2.4	–	3.0	2.6	1.83
	SSP-PA-08	1.9	4.8	–	2.0	5.0	1.13
	SSP-LA-01	–	4.7	2.0	3.0	4.5	2.0
	SSP-LA-05	–	1.5	1.5	1.4	1.67	1.5
Wastewater pond in seafood processing plant (SC)	SC-PA-03	5.0	2.0	–	4.33	1.33	2.0
	SC-PA-08	3.0	1.5	–	3.25	1.33	1.2
	SC-PAL-02	2.25	1.0	1.5	2.25	1.17	1.4
Aerobic treatment pond in seafood processing plant (HP)	HP-PA-01	4.0	1.0	–	4.0	0.8	2.0
	HP-LA-11	–	0.7	0.5	1.2	0.71	0.5
	HP-LA-12	–	2.0	0.5	0.33	2.0	0.5
	HP-PAL-01	0.50	1.0	0.6	0.63	1.0	0.57

PA: protease/amylase medium, LA: lipase/amylase medium, PAL: protease/amylase/lipase medium, PI: proteolytic index, AI: amylolytic index, LI: lipolytic index. –: not determined.

Agro-Industry, Prince of Songkla University. These include *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus* sp. B.24, *Bacillus* sp. LN008, and *Bacillus* sp. MK007. It should be noted that *Bacillus* species are frequently used as beneficial microorganisms in aquaculture. They are preferred strains for enzyme production due to their robust growth and their ability to adapt to varying growth conditions [12]. The cultivations were conducted at 30 ± 2°C with a shaking speed of 150 rpm for 48 h. The results are shown in Fig. 1. Eleven isolates, including SSP-PA-08, SC-PA-03, SC-PA-08, SC-PAL-02, HP-PA-01, HP-LA-11, HP-LA-12, HP-PAL-01, *B. coagulans*, *Bacillus* sp. MK007, and *B. subtilis*, exhibited relatively high protease, lipase, and amylase abilities compared to other isolates. The isolate SC-PA-08 showed the highest protease activity of 6.23 ± 0.81 U/mL, followed by isolates SSP-PA-08 and HP-PA-01, which exhibited protease activities of 4.53 ± 0.24 and 4.28 ± 0.11 U/mL, respectively. While *Bacillus* sp. MK007 exhibited the highest amylase activities of 2.01 ± 0.01 U/mL, HP-PAL-01 exhibited the highest lipase productivity of 0.25 ± 0.14 U/mL. Among the strains screened, SC-PA-03, SC-PA-08, SC-PAL-02, HP-PA-01, HP-LA-11, HP-LA-12, and HP-PAL-01 exhibited high dual hydrolytic activities of protease and lipase. Noteworthy, SSP-PA-08, *B. coagulans* and *Bacillus* sp. MK007, demonstrated high triple hydrolytic activities of protease, amylase, and lipase. In this study, the bacterial strains that showed considerably high multiple hydrolytic activities were selected to study enzyme production using synthetic shrimp pond sludge as a low-cost nutrient source and also their bioremediation potential.

3.2.2. Tertiary quantitative screening using synthetic shrimp pond sludge

The accumulation of sludge occurs due to excessive use of feed in intensive aquaculture and waste from farmed aquatic animals. In general, TSS in shrimp ponds is mainly derived from residual shrimp feed and present at concentrations of 0.8–2.7%. This high TSS causes a negative impact on water quality [20]. This study aimed at utilizing synthetic shrimp pond sludge as a low-cost medium for the production of hydrolytic enzymes and also evaluating the bioremediation potential of bacteria to reduce TSS. Each selected bacterium was inoculated into synthetic shrimp pond sludge containing TSS of 0.8% at an initial inoculum size of 10^8 CFU/mL. The medium pH was adjusted to 7. As shown in Fig. 2, it was found that all strains exhibited the highest enzyme activities at 24 h. Among them, SC-PAL-02 exhibited the maximum protease activity of 4.83 ± 0.55 U/mL followed by SC-PA-03 and SC-PA-08, which showed protease activities of 3.84 ± 0.49 U/mL and 3.63 ± 0.19 U/mL, respectively. Regarding amylase activity, the bacterial strain *B. coagulans* had the highest amylase activity of 3.50 ± 0.41 U/mL, followed by SC-PA-08 (2.10 ± 1.21 U/mL). Regarding lipase activity, HP-LA-11 demonstrated the highest lipase activity of 0.41 ± 0.06 U/mL, followed by *Bacillus* sp. MK007 (0.29 ± 0.08 U/mL). The reduction of enzyme activity at 48 h might be due to the depletion of nutrients or the bacteria having achieved sufficient enzymatic activity to digest organic substances [21].

When comparing the TSS reduction, SSP-PA-08 was most effective in reducing TSS by 60.16 ± 1.37%, followed by HP-PA-01, *Bacillus* sp. MK007, and *B. coagulans*, which reduced TSS by 56.64 ± 0.64%, 53.04 ± 1.28%, and 51.63 ± 1.07%, respectively (Fig. 2d). The ability of these isolates to reduce TSS indicated their potential application in sludge bioremediation [22]. Based on these results, it could be concluded that the enzyme-producing ability of the bacteria depends largely on the type of medium (agar and liquid) and the mix of substrates. As this study aimed at producing multienzymes using low-cost shrimp pond sludge medium and bioremediation of shrimp pond sludge, SC-PAL-02, HP-LA-11, and *B. coagulans,* those exhibited the highest protease, lipase, and amylase activities, respectively, and SSP-PA-08 that was most capable of reducing TSS > 60%, were selected for further studies.

3.3. Identification of bacterial strains based on 16S rRNA sequences

The newly isolated SC-PAL-02, HP-LA-11, and SSP-PA-08 were subjected to identification by 16S rDNA sequencing. The partial 16S rDNA sequences of isolates SC-PAL-02, HP-LA-11, and SSP-PA-08 were submitted to GenBank. BLAST analysis revealed that the partial 16S rRNA gene sequences of isolates SC-PAL-02 and HP-LA-11 showed 95% similarity to *Aeromonas* sp., while isolate SSP-PA-08 exhibited 97% similarity to *Exiguobacterium indicum*. However, *Aeromonas* sp. was classified as pathogenic bacteria.

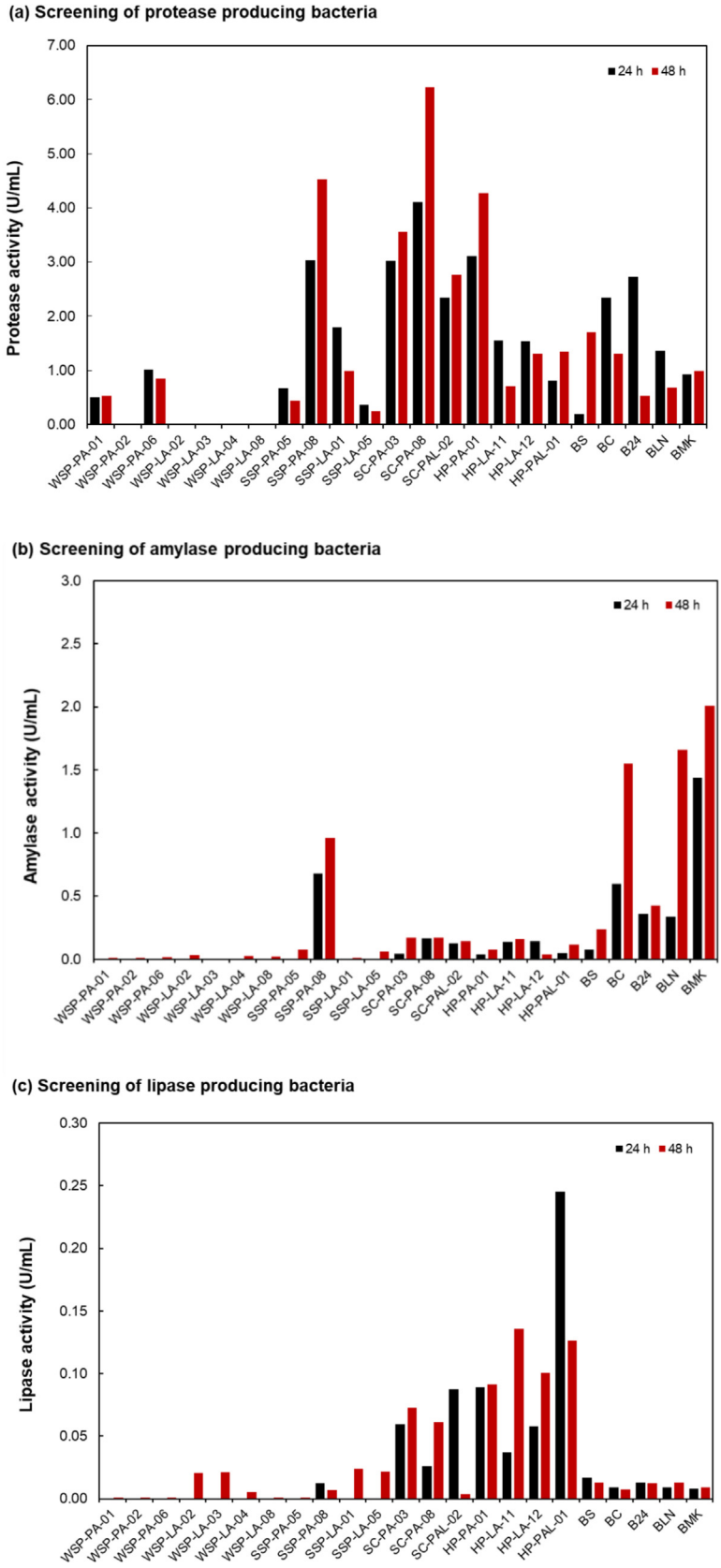

Fig. 1. Enzyme activities of isolates cultured in (a) skim milk broth medium, (b) starch broth medium and (c) Tween 80 broth medium. BS: *Bacillus subtilis*; BC: *Bacillus coagulans*; B24: *Bacillus* sp. B.24; BLN: *Bacillus* sp. LN008; BMK: *Bacillus* sp. MK007. The cultivations were conducted at 30 ± 2°C with a shaking speed of 150 rpm for 48 h.

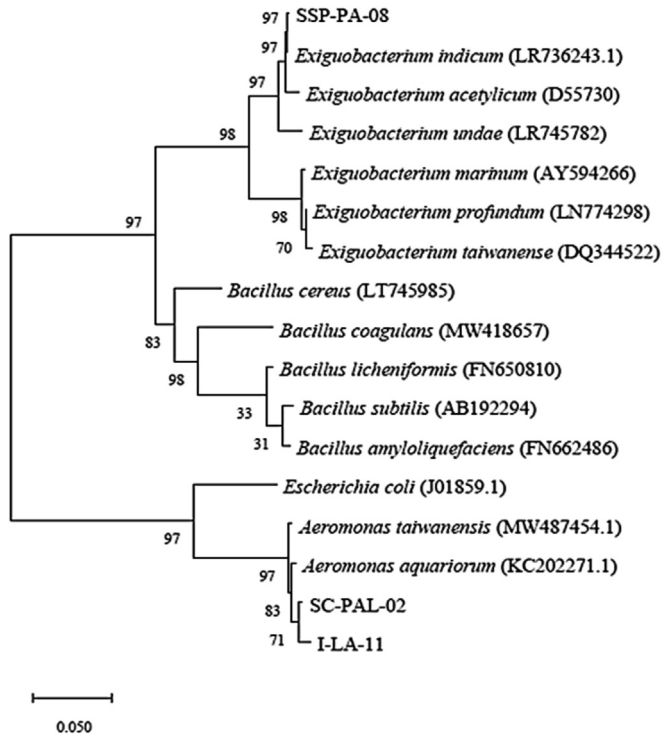

Fig. 2. Enzyme activity and total suspended solid (TSS) removal by isolates cultured in synthetic shrimp pond sludge medium. BC: *Bacillus coagulans*; BMK: *Bacillus* sp. MK007; BS: *Bacillus subtilis*. Each bacterium was inoculated in synthetic shrimp pond sludge containing TSS of 0.8% at an initial inoculum size of 10^8 CFU/mL. The cultivations were conducted at 30 ± 2°C with a shaking speed of 150 rpm for 48 h.

Therefore, *E. indicum* SSP-PA-08 with secondly high protease producing ability and most capable of reducing TSS was selected. The sequence data were deposited in the DDBJ database under GenBank accession number LC645459. The 16S rRNA gene sequence of *E. indicum* SSP-PA-08, along with sequences from closely related representative species retrieved from GenBank, was aligned using the Clustal W program. The maximum likelihood technique was used to reconstruct a phylogenetic tree. Evolutionary distances were determined using the Jukes-Cantor method. To assess the confidence limits of the branching, the bootstrap analysis was performed. Fig. 3 depicts the phylogenetic tree showing the relationship among SSP-PA-08 strains and their phylogenetically closest strains. The maximum likelihood phylogenetic tree demonstrated that strain SSP-PA-08 forms a distinct lineage with the strain type of *E. indicum*. Similarly, Kumar et al. [23] found that a protease-producing bacterium, TBG-PICH-001, isolated from soil in Pichavaram estuary, Tamil Nadu, was identified as *E. indicum*. Nevertheless, Yasin et al. [24] found that *Exiguobacterium* sp. strain AMBL-20 T isolated from ice in the northeastern region of Pakistan was capable of producing lipase, which showed potential use as a bio-detergent.

3.4. Enzyme production using synthetic shrimp pond sludge

3.4.1. Mono- and co-cultures

Hydrolytic enzyme production by mono- and co–cultures of the selected bacterial strains was attempted by cultivating in a synthetic shrimp pond sludge medium. The study was conducted using a synthetic sludge medium containing TSS of 0.8%, with an initial pH of 7.0 and incubated on a shaker at 150 rpm at 30 ± 2°C for 72 h. Fig. 4 shows that the mono-culture of SC-PAL-02 exhibited the highest protease activity of 4.83 ± 0.55 U/mL at 24 h, while the mono-culture of *B. coagulans* exhibited the highest amylase activity of 3.5 ± 0.41 U/mL at 24 h. The mono-culture of HP-LA-11 showed the highest lipase activity of 0.41 ± 0.06 U/mL at 24 h. However, when the co-culture of three selected strains (PAL-01) was performed, the results showed that the co-culture yielded lower activities of each enzyme. The protease, amylase, and lipase activities were reduced to 2.3 ± 1.5 U/mL, 0.77 ± 0.05 U/mL, and 0.18 ± 0.06 U/mL, respectively. It was possible that each bacterial strain might compete for nutrients and/or there might be an antagonistic interaction [23]. Nevertheless, the co-culture most reduced TSS by 62.5 ± 0.4% despite the lower individual enzyme activity than the mono-culture. It was possible that individual bacteria might have evolved different mechanisms to utilize the substrates, such as breaking down larger organic particles into smaller ones, thereby increasing the surface area available for biodigestion of organic matter [25,26]. Ramu et al. [27] also found that the co-culture of *B. subtilis*, *B. licheniformis*, and *B. firmus*, which possessed amylase, protease, and cellulase activities, could promote the biodegradation of organic matter in a shrimp pond.

In addition to the mono- and co-cultures of the bacterial strains with the highest hydrolytic activities, the second strain that produced high protease activity, *Exiguobacterium indicum* SSP-PA-08 (EI), and that produced high amylase and lipase activities, *B. coagulans* and *B. subtilis*, were selected for the co-culture. It should be noted that *E. indicum* has also been reported for its probiotic properties in shrimp [28]. Additionally, Hakim et al. [29] reported that *E. indicum* AKAL11 could produce high alkaline protease enzymes using municipal solid waste as a medium. Additionally, the *Bacillus* group has also been reported for their probiotic capabilities, including their ability to bind and provide immunostimulant [30]. In the second run of co-culture, the mono- and co-cultures of EI with *B. coagulans* and *B. subtilis* were performed using synthetic shrimp pond sludge medium at a TSS concentration of 0.8%. The co-culture resulted in greater enzyme production compared to the mono-culture of EI (Fig. 5). When the medium used for enzyme production contains a variety of nutrients such as proteins, starches, and fats, microorganisms may develop various mechanisms that enable complex nutrient utilization and enhance the production of individual enzymes [25]. Moreover, co-culturing bacteria can also share metabolic activities, i.e., multiple enzymes that synergistically facilitate the sequential degradation and exchange of metabolites [25,31]. Cao et al. [32] revealed that microbial consortia are more effective at degrading complex organic matter than isolated bacteria. They are also more adaptable and stable within the growth environment and can offer a suitable catalytic environment for essential enzymes involved in the biodegradation pathway. The study demonstrated that the activities of these three key enzymes were crucial for effective wastewater treatment.

The bioremediation potential of the mono- and co-cultures is shown in Fig. 6. It was found that the TSS reduction and protein solubilization by mono- and co-cultures were not significantly different, while the COD removal by the co-culture was higher than that by the mono-culture. This study demonstrates that co-culturing EI with *B. coagulans* and *B. subtilis* not only promoted the synergistic enzyme production but also the biodegradation of organic matter. Furthermore, the biodegradation process also increased the number of microorganisms and their metabolic activities. The more microorganisms grew, the more enzymes were produced. Hence, bacterial enzymatic reactions gradually solubilize complex compounds into simpler and easier for assimilation [27,33].

3.4.2. Effect of synthetic sludge concentration

Fig. 5 also shows the effect of synthetic sludge concentration on enzyme production by the co-culture compared with the monoculture of *E. indicum*. The study was conducted using a synthetic

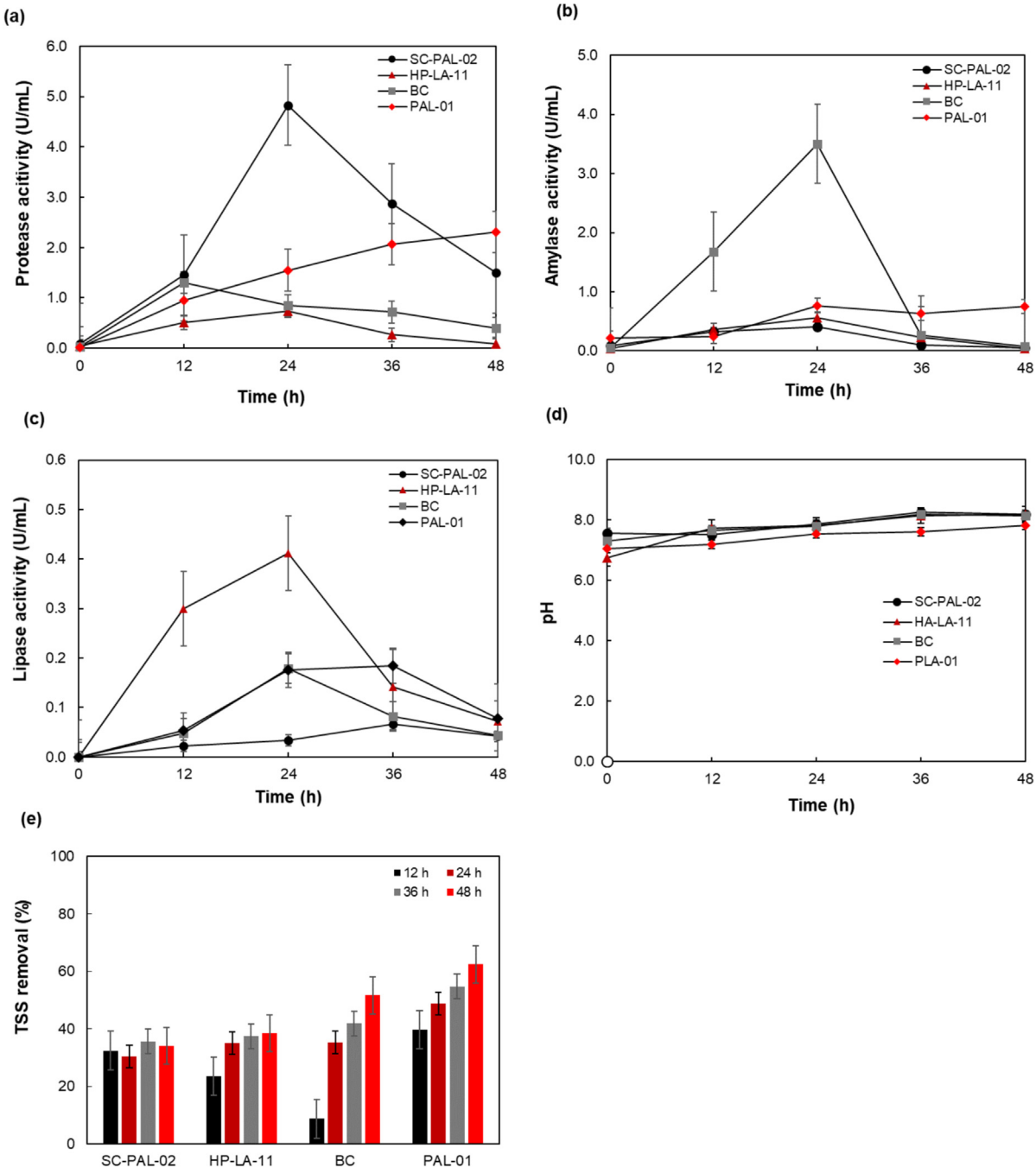

Fig. 3. The phylogenetic tree showing the relationship among SSP-PA-08 strains and their phylogenetically closest strains.

sludge medium containing 0.8, 2.0, and 2.7% TSS, with an initial pH of 7.0 and incubated on a shaker at 150 rpm at 30 ± 2°C for 72 h. The co-culture using synthetic shrimp pond sludge medium at 0.8% TSS exhibited the highest protease activity of 1.81 ± 0.44 U/mL at 48 h. Increasing concentrations of sludge decreased protease production. Although co-culturing bacteria can facilitate shared metabolic activities that enable complex nutrient utilization [25,31], excessively high concentrations of sludge also reduce mass transfer and oxygen availability. This thereby may inhibit bacterial growth and enzyme production [34]. Similarly, Kandasamy et al. [34], who studied the protease production using different loadings of coffee pulp waste (CPW) and corncob (CC), found that the optimum substrate loading was 0.3% CPW and 0.2% CC. The further increase of solid substrate than the optimal level decreased the protease production. Additionally, the pH of mono-culture of *E. indicum* decreased over the first 12 h and then increased, whereas the co-culture maintained a relatively constant pH (data not shown). This pH stability in the co-culture can influence both enzyme production and activities.

Fig. 6 shows the effect of sludge concentration on the number of soluble proteins, TSS, and COD. Increasing sludge concentration led to a corresponding increase in initial soluble protein, TSS, and COD. It was found that the co-culture reduced the amount of TSS and soluble COD better than the mono-culture possibly due to the synergistic effects of multienzyme activities that could accelerate the decomposition of solid sludge. The decomposition of large organic molecules releases soluble molecules and enables bacteria to assimilate organic matter more efficiently. When increasing the

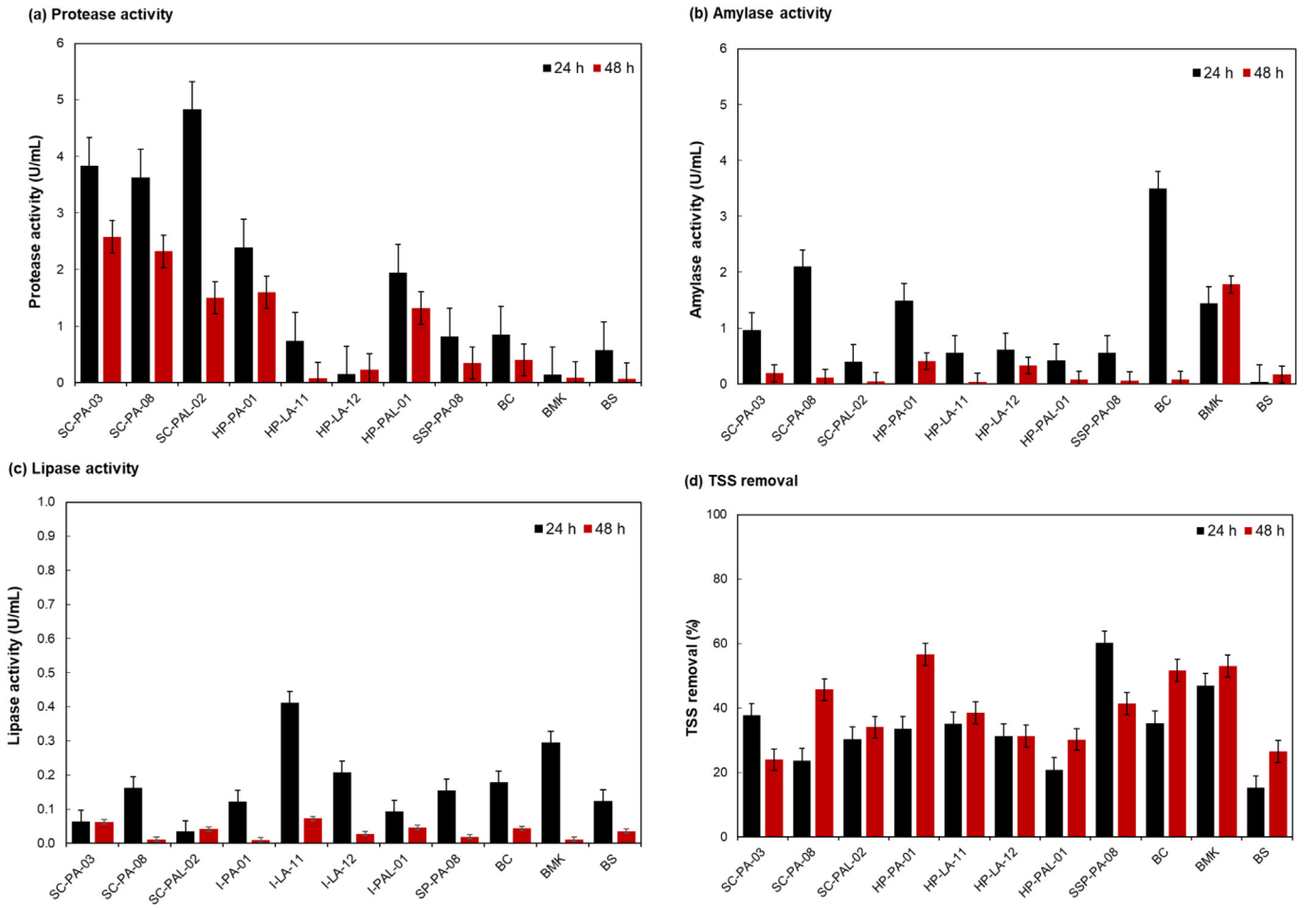

Fig. 4. Enzyme production, pH and total suspended solid (TSS) removal (%) by pure and co-culture of selected bacteria. PAL-01 is the co-culture with the same inoculum portion (10^8 CFU/mL). (a) Protease activity; (b) Amylase activity; (c) Lipase activity; (d) pH; (e) TSS removal. The synthetic sludge medium contained TSS of 0.8%. The cultivations were conducted at 30 ± 2°C with a shaking speed of 150 rpm for 72 h.

sludge concentration from 0.8% to 2.0% and 2.7%, the COD removal efficiencies reduced from 67.39 ± 1.21% to 68.75 ± 0.61% and 34.78 ± 1.21%, respectively.

3.5. Optimization of enzyme production using shrimp pond sludge

In this study, the shrimp pond sludge wastewater was used for the cultivation of co-culture for enzyme production and evaluation of bioremediation potential. The effect of sludge concentration at 0.6, 1.0, 1.6, and 2.8% was studied (Fig. 7). The sludge concentration of 1.6% resulted in the highest production of protease and amylase enzymes of 0.54 ± 0.06 and 1.01 ± 0.06 U/mL, respectively. When comparing the enzyme production by using shrimp pond sludge wastewater to synthetic sludge wastewater, it was observed that protease and amylase enzyme production in shrimp pond sludge wastewater was much lower. The enzyme production decreased when the sludge concentration was increased up to 2.8%, likely due to mass and oxygen transfer limitations that occur at high solid substrate loadings. During the culture in sludge wastewater, it was observed that the pH remained relatively stable, ranging between 7 and 8 (data not shown). With increasing the sludge concentration, the concentration of soluble protein also increased. The soluble protein decreased slightly during the first 24 h of fermentation. Additionally, both TSS and soluble COD decreased over time, which was attributed to the activity of the three bacterial strains. Nutrients play a crucial role in promoting bacterial growth, enabling the bacteria to oxidize COD and release carbon dioxide [35]. The highest enzyme production was obtained when using shrimp pond sludge wastewater that contained 1.6% TSS. However, the TSS concentration was still high in the culture broth. This might be due to some compounds in the sludge wastewater not being efficiently utilized as substrates by the metabolic mechanisms of enzyme-producing microorganisms [36]. The treated aquaculture sludge might be used as a potential organic fertilizer [37].

4. Conclusions

This study has developed the methods to isolate bacteria with multienzyme-producing ability. From primary and secondary quantitative screening, eleven bacterial strains demonstrating substantial production of protease, amylase, and lipase were selected. From tertiary quantitative screening, the newly isolated *E. indicum* (SSP-PA-08) was chosen as a high protease producer, while *B. coagulans* and *B. subtilis* were selected as amylase and lipase producers, respectively. Co-culturing these bacteria in synthetic shrimp pond sludge significantly enhanced protease activity by 2.5 folds and amylase activity by 20 folds and led to a greater reduction in total suspended solids compared to the mono-culture possibly due to the synergistic effects of multienzyme activities that could accelerate the decomposition of solid sludge. This study also showed that it was possible to use shrimp pond sludge for enzyme production

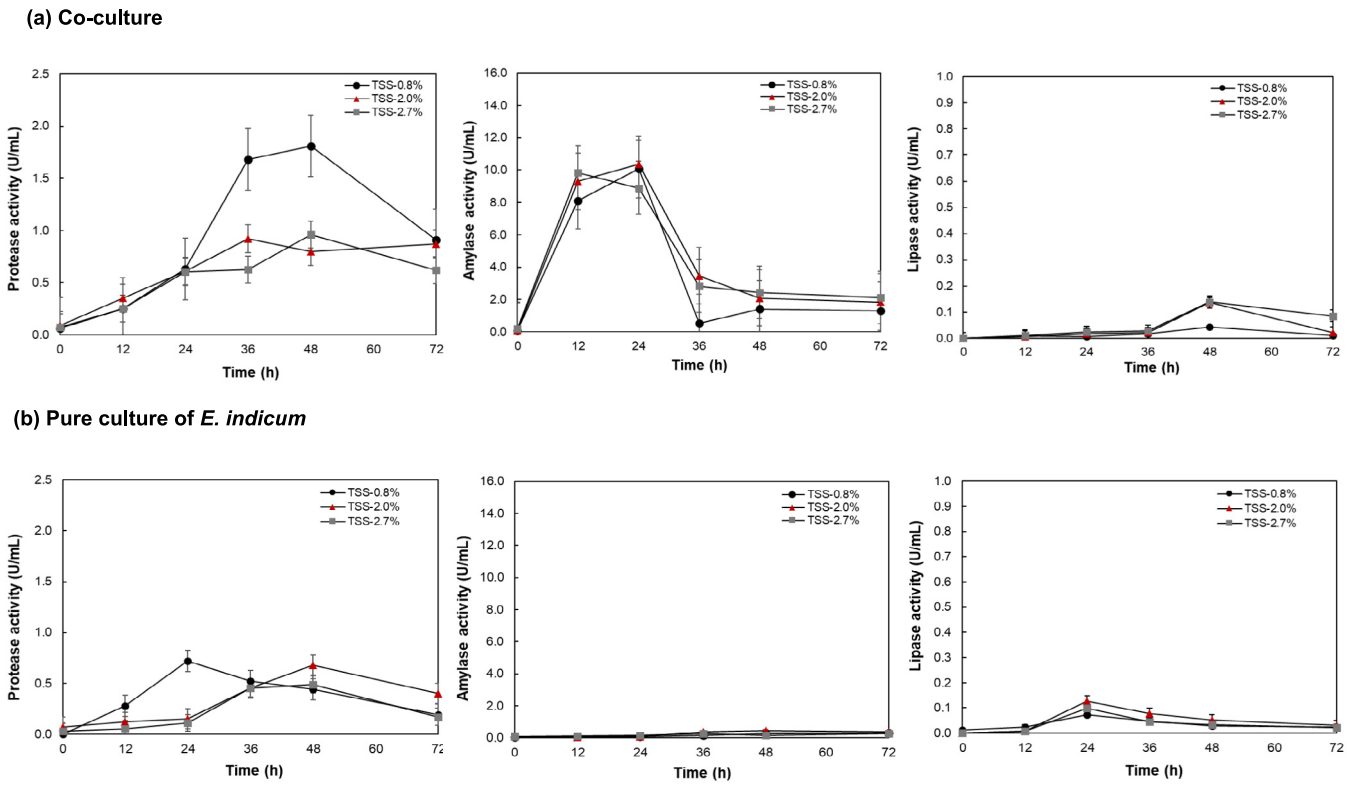

Fig. 5. Effect of sludge concentration on enzyme production by co-culture (PAL) of protease-producing *E. indicum* (P) with amylase-producing *B. coagulans* (A) and lipase-producing *B. subtilis* (L) compared with pure culture of *E. indicum*. The cultivations were conducted at 30 ± 2°C with a shaking speed of 150 rpm for 72 h.

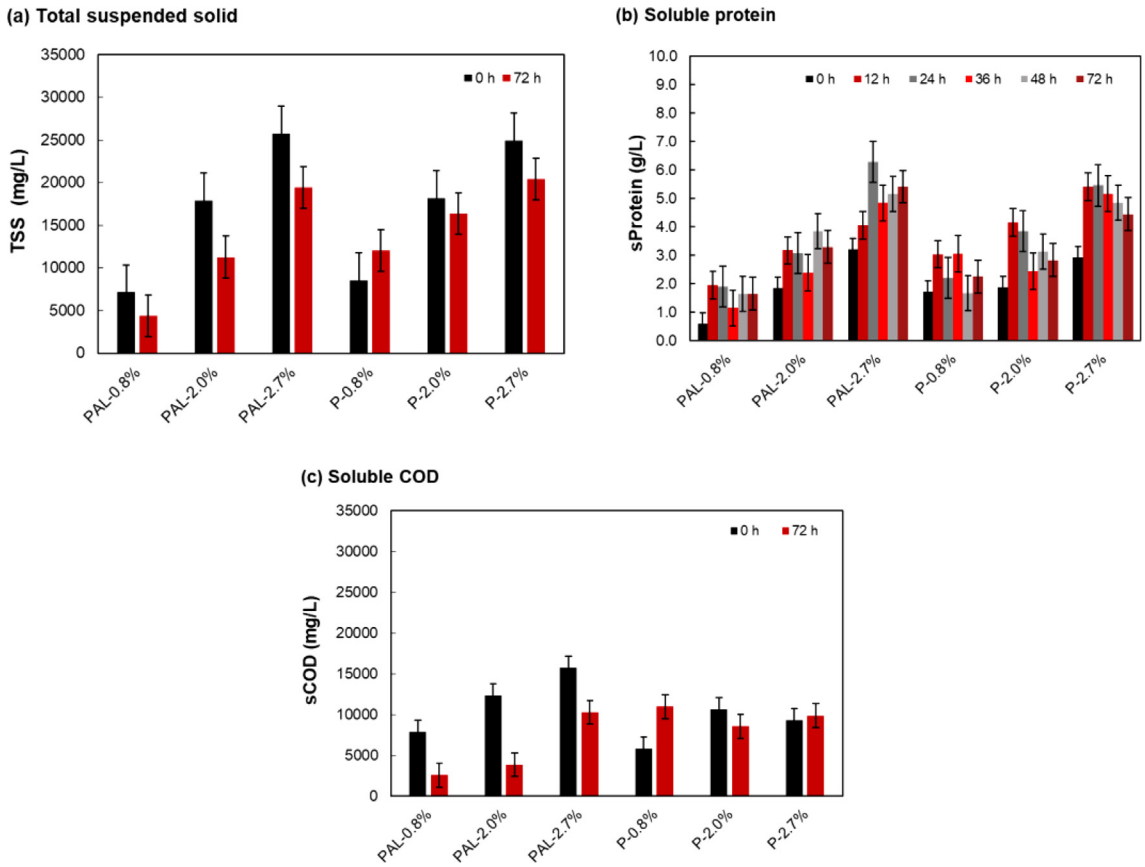

Fig. 6. Effect of sludge concentration at 0.8%, 2.0% and 2.7% on soluble protein (sProtein), total suspended solid (TSS) and soluble COD (sCOD) by co-culture (PAL) of protease-produce *E. indicum* (P) with amylase-produce *B. coagulans* (A) and lipase-produce *B. subtilis* (L) compared with pure culture of *E. indicum* (P). The cultivations were conducted at 30 ± 2°C with a shaking speed of 150 rpm for 72 h.

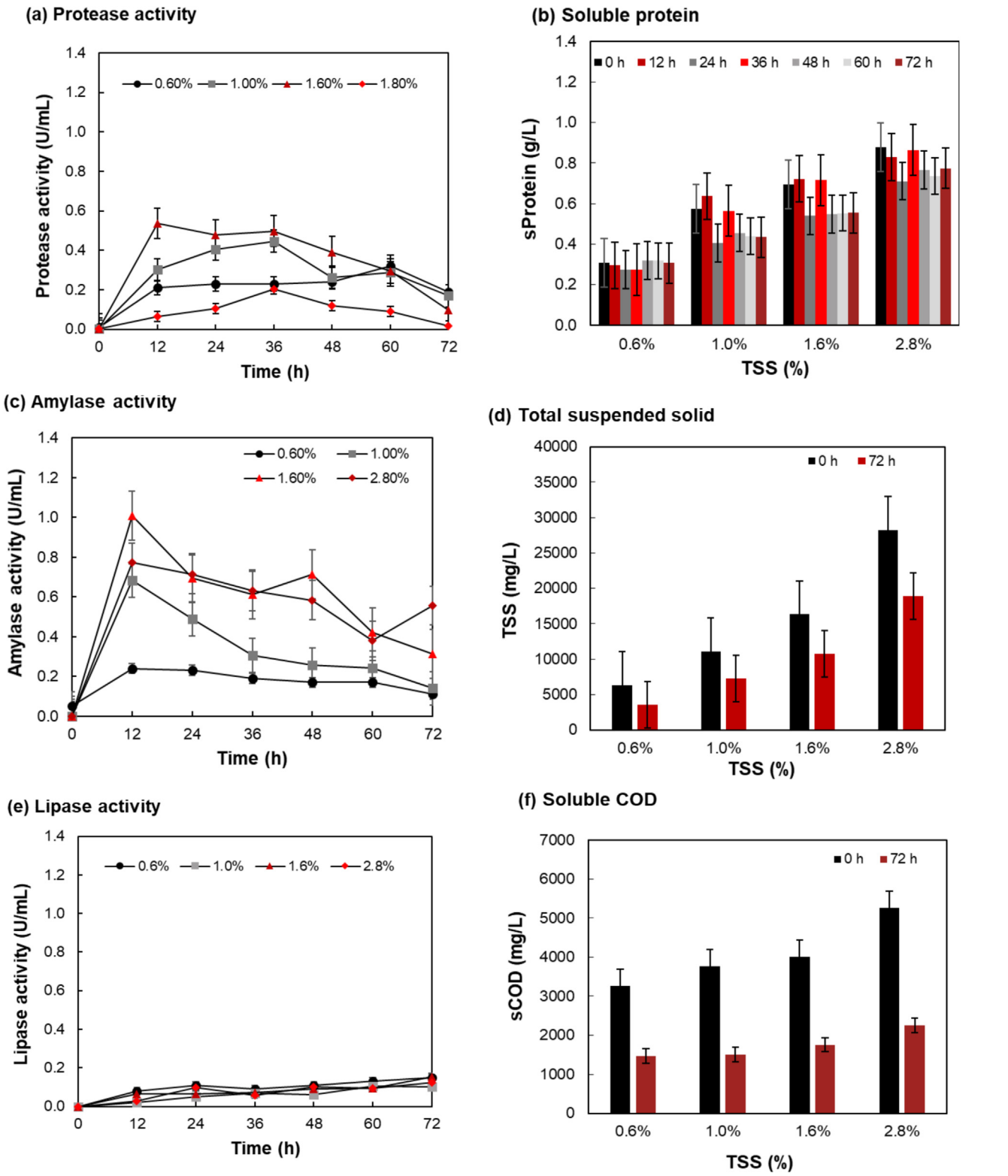

Fig. 7. Effect of sludge concentration on enzyme activities, soluble protein (sProtein), total suspended solid (TSS) and soluble COD (sCOD) using shrimp pond wastewater for 72 h. The cultivations were conducted at 30 ± 2°C with a shaking speed of 150 rpm for 72 h.

and the highest enzyme production was recorded when using shrimp pond sludge containing 1.6% TSS. These strategies have shown that it is possible to isolate and synthesize effective bacterial consortia as an environmentally friendly solution to treat shrimp pond sludge and may be possible to apply it to other aquaculture sludge.

CRediT authorship contribution statement

Chutema Thongsongkaew: Methodology, Investigation, Writing – original draft, Data curation. **Benjamas Cheirsilp:** Methodology, Funding acquisition, Supervision, Writing – review & editing, Conceptualization. **Asma Billateh:** Writing – original draft, Data curation. **Wageeporn Maneechote:** Methodology, Writing – original draft. **Sirasit Srinuanpan:** Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary material

https://doi.org/10.1016/j.ejbt.2025.05.005.

Data availability

Data will be made available on request.

References

[1] Jasmin MY, Syukri F, Kamarudin MS, et al. Potential of bioremediation in treating aquaculture sludge. Aquaculture 2020;519:734905. https://doi.org/10.1016/j.aquaculture.2019.734905.

[2] Kamal SZ, Koyama M, Syukri F, et al. Effect of enzymatic pre-treatment on thermophilic composting of shrimp pond sludge to improve ammonia recover. Environ Res 2022;204:112299. https://doi.org/10.1016/j.envres.2021.112299. PMid: 34743806.

[3] Iber BT, Kasan NA. Recent advances in shrimp aquaculture wastewater management. Heliyon 2021;7(11):e08283. https://doi.org/10.1016/j.heliyon.2021.e08283. PMid: 34778576.

[4] Tom AP, Jayakumar JS, Biju M, et al. Aquaculture wastewater treatment technologies and their sustainability: A review. Energy Nexus 2021;4:100022. https://doi.org/10.1016/j.nexus.2021.100022.

[5] Ghattavi S, Homaei A. Marine enzymes: Classification and application in various industries. Int J Biol Macromolecules 2023;230:123136. https://doi.org/10.1016/j.ijbiomac.2023.123136. PMid: 36621739.

[6] Sharifian S, Homaei A, Kim SK, et al. Production of newfound alkaline phosphatases from marine organisms with potential functions and industrial applications. Process Biochem 2018;64:103–15. https://doi.org/10.1016/j.procbio.2017.10.005.

[7] Izadpanah Qeshmi F, Homaei A, Khajeh K, et al. Production of a novel marine *Pseudomonas aeruginosa* recombinant L-asparaginase: Insight on the structure and biochemical characterization. Mar Biotechnol 2022;24:599–613. https://doi.org/10.1007/s10126-022-10129-9. PMid: 35507234.

[8] Gómez S, Hurtado CF, Orellana J. Bioremediation of organic sludge from a marine recirculating aquaculture system using the polychaete *Abarenicola pusilla* (Quatrefages, 1866). Aquaculture 2019;507:377–84. https://doi.org/10.1016/j.aquaculture.2019.04.033.

[9] Shaik M, Sanker GG, Iswarya M, et al. Isolation and characterization of bioactive metabolites producing marine *Streptomyces parvulus* strain sankarensis-A10. J Genet Eng Biotechnol 2017;15(1):87–94. https://doi.org/10.1016/j.jgeb.2017.02.004. PMid: 30647645.

[10] Gozari M, Mortazavi MS, Bahador N, et al. Isolation and screening of antibacterial and enzyme-producing marine actinobacteria to approach probiotics against some pathogenic vibrios in shrimp *Litopenaeus vannamei*. Iran J Fish Sci 2016;15(2):630–44.

[11] Zottig X, Meddeb-Mouelhi F, Beauregard M. Development of a high-throughput liquid state assay for lipase activity using natural substrates and rhodamine B. Anal Biochem 2016;496:25–9. https://doi.org/10.1016/j.ab.2015.11.020. PMid: 26706798.

[12] Xu X, Nielsen LJD, Song L, et al. Enhanced specificity of *Bacillus metataxonomics* using a *tuf*-targeted amplicon sequencing approach. ISME Commun 2023;3(1):126. https://doi.org/10.1038/s43705-023-00330-9. PMid: 38012258.

[13] Ruangwicha J, Cheirsilp B, Suyotha W. Green biorefinery of shrimp shell waste for α-chitin and high-value co-products through successive fermentation by co-lactic acid bacteria and proteolytic fungus. Bioresour Technol 2024;393:130106. https://doi.org/10.1016/j.biortech.2023.130106. PMid: 38008224.

[14] Jain A, Jain R, Jain S. Quantitative analysis of reducing sugars by 3, 5-Dinitrosalicylica acid (DNSA Method). In: Basic techniques in biochemistry, microbiology and molecular biology. Springer protocols handbooks. New York, NY: Humana; 2020. https://doi.org/10.1007/978-1-4939-9861-6_43.

[15] Louhasakul Y, Cheirsilp B. Potential use of industrial by-products as promising feedstock for microbial lipid and lipase production and direct transesterification of wet yeast into biodiesel by lipase and acid catalysts. Bioresour Technol 2022;348:126742. https://doi.org/10.1016/j.biortech.2022.126742. PMid: 35065222.

[16] Ibrahim ASS, Al-Salamah AA, Elbadawi YB, et al. Production of extracellular alkaline protease by new halotolerant alkaliphilic *Bacillus* sp. NPST-AK15 isolated from hyper saline soda lakes. Electron J Biotechnol 2015;18(3):236–43. https://doi.org/10.1016/j.ejbt.2015.04.001.

[17] Kot AM, Błażejak S, Kurcz A, et al. Biodegradation of deproteinized potato wastewater and glycerol during cultivation of *Rhodotorula glutinis* yeast. Electro J Biotechnol 2015;18(6):428–32. https://doi.org/10.1016/j.ejbt.2015.08.006.

[18] Panigrahi A, Saranya C, Sundaram M, et al. Carbon, nitrogen (C/N) ratio level variation influences microbial community of the system and growth as well as immunity of shrimp (*Litopenaeus vannamei*) in biofloc based culture system. Fish Shellfish Immunol 2018;81:329–37. https://doi.org/10.1016/j.fsi.2018.07.035. PMid: 30016684.

[19] Al-Dhabi NA, Esmail GA, Mohammed Ghilan AK, et al. Isolation and screening of *Streptomyces* sp. Al-Dhabi-49 from the environment of Saudi Arabia with concomitant production of lipase and protease in submerged fermentation. Saudi J Biol Sci 2020;27(1):474–9. https://doi.org/10.1016/j.sjbs.2019.11.011. PMid: 31889873.

[20] Gaona CAP, de Almeida MS, Viau V, et al. Effect of different total suspended solids levels on a *Litopenaeus vannamei* (Boone, 1931) BFT culture system during biofloc formation. Aquac Res 2017;48(3):1070–9. https://doi.org/10.1111/are.12949.

[21] Solangi F, Zhu X, Solangi KA, et al. Responses of soil enzymatic activities and microbial biomass phosphorus to improve nutrient accumulation abilities in leguminous species. Sci Rep 2024;14:11139. https://doi.org/10.1038/s41598-024-61446-z. PMid: 38750151.

[22] Soaudy MR, Ghonimy A, Greco LSL, et al. Total suspended solids and their impact in a biofloc system: Current and potentially new management strategies. Aquaculture 2023;572:739524. https://doi.org/10.1016/j.aquaculture.2023.739524.

[23] Kumar SS, Jithin V, Jijeesh V, et al. Production and purification of alkaline protease from *Exiguobacterium indicum* TBG-PICH-001 isolated from soil samples of Pichavaram Estuary (Tamil Nadu). Indian J Geo-Mar Sci 2018;47(3):580–6.

[24] Yasin MT, Ali Y, Ahmad K, et al. Alkaline lipase production by novel mesotolerant psychrophilic *Exiguobacterium* sp. strain (AMBL-20) isolated from glacier of northeastern Pakistan. Arch Microbiol 2021;203(4):1309–20. https://doi.org/10.1007/s00203-020-02133-1. PMid: 33325000.

[25] Ramamurthy V, Cheepurupalli L, Singh SR, et al. Co-culture:A promising method in enzyme production. Int J ChemTech Res 2017;10(6):720–6.

[26] Saelor A, Kongjan P, Prasertsan P, et al. Enhancing the efficiency of high solid anaerobic digestion of empty fruit bunches under thermophilic conditions by particle size reduction and co-digestion with palm oil mill effluent. Carbon Resour Conver 2024;8(2):100262. https://doi.org/10.1016/j.crcon.2024.100262.

[27] Ramu D, Sigamani S, Venkatachalam H, et al. The role of probiotics in the control of bacterial diseases and biodegradation of organic matter in shrimp (*Penaeus vannamei*) culture ponds of South India. J Coastal Life Med 2017;5(7):293–8. https://doi.org/10.12980/jclm.5.2017J7-32.

[28] Cong M, Jiang Q, Xu X, et al. The complete genome sequence of *Exiguobacterium arabatum* W-01 reveals potential probiotic functions. MicrobiologyOpen 2017;6(5):e00496. https://doi.org/10.1002/mbo3.496. PMid: 28589562.

[29] Hakim A, Bhuiyan FR, Iqbal A, et al. Production and partial characterization of dehairing alkaline protease from *Bacillus subtilis* AKAL7 and *Exiguobacterium indicum* AKAL11 by using organic municipal solid wastes. Heliyon 2018;4(6). https://doi.org/10.1016/j.heliyon.2018.e00646. PMid: 30009270.

[30] Bahaddad SA, Almalki MHK, Alghamdi OA, et al. *Bacillus* species as direct-fed microbial antibiotic alternatives for monogastric production. Probiotics Antimicrob Proteins 2023;15(1):1–16. https://doi.org/10.1007/s12602-022-09909-5. PMid: 35092567.

[31] Lázaro-Mass S, Gómez-Cornelio S, Castillo-Vidal M, et al. Biodegradation of hydrocarbons from contaminated soils by microbial consortia: A laboratory microcosm study. Electron J Biotechnol 2023;61:24–32. https://doi.org/10.1016/j.ejbt.2022.10.002.

[32] Cao Z, Yan W, Ding M, et al. Construction of microbial consortia for microbial degradation of complex compounds. Front Bioeng Biotechnol 2022;10 (1):1051233. https://doi.org/10.3389/fbioe.2022.1051233. PMid: 36561050.
[33] Safitri R, Priadie B, Miranti M, et al. Ability of bacterial consortium: *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus subtilis*, *Nitrosomonas* sp., and *Pseudomonas putida* in bioremediation of wastewater in Cisirung wastewater treatment plant. AgroLife Sci J 2015;4(1):146–52.
[34] Kandasamy S, Muthusamy G, Balakrishnan S, et al. Optimization of protease production from surface-modified coffee pulp waste and corncobs using *Bacillus* sp. by SSF. 3 Biotech 2016;6:167. https://doi.org/10.1007/s13205-016-0481-z. PMid: 28330239.
[35] Sial A, Zhang B, Zhang A, et al. Microalgal–bacterial synergistic interactions and their potential influence in wastewater treatment: A review. Bioenergy Res 2021;14(3):723–38. https://doi.org/10.1007/s12155-020-10213-9.
[36] Nzila A, Razzak SA, Zhu J. Bioaugmentation, an emerging strategy of industrial wastewater treatment for reuse and discharge. Int J Environ Res Public Health 2016;13(9):846. https://doi.org/10.3390/ijerph13090846. PMid: 27571089.
[37] Madariaga ST, Marín SL. Sanitary and environmental conditions of aquaculture sludge. Aquac Res 2017;48(4):1744–50. https://doi.org/10.1111/are.13011.