

***Bacillus* Multi-strain from Malaysian Fish Sauces Demonstrating Proteolytic, Lipolytic, Esterolytic and Glutamic-acid Production Activities**

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Abstract

Background and Objective: Aroma and taste of fish sauce are derived from complex metabolic reactions involving bacteria and enzymes that degrade proteins and lipids. A longer fermentation time is needed to fully develop the rich flavor. The objective of this study was to isolate halotolerant bacteria from Malaysian fermented fish sauce that demonstrated significant proteolytic, lipolytic, esterolytic and glutamic acid-accumulating activities. These bacteria might potentially be used as starting cultures to enhance development of flavors during fish sauce fermentation.

Material and Methods: The initial screening on proteolytic activity was carried out on saline skim milk agar. Isolates with high proteolytic index and positive lipase/esterase activity were further assessed using casein-based protease activity assay. Protease and lipase activities under various conditions of strains, NaCl concentrations (20–30% w/v), temperatures (30–35 °C) and incubation times (0–120 h) were assessed and analyzed using four-way ANOVA. The p-nitrophenyl butyrate and colorimetric assays were used to determine esterase and glutamic acid accumulating activities, respectively.

Results and Conclusion: Six strains isolated from budu demonstrated hydrolytic activities and identified as *Bacillus* spp. Proteolytic activities were averagely highest in *B. licheniformis* 12N3A at 25% NaCl, 30 °C and 96 h of incubation. The highest lipolytic activities were achieved with *B. haikouensis* 12M1F at 30% NaCl, 35 °C and 72 h of incubation. *Bacillus* sp. 3M3A showed the highest esterase activity of 155.48 U, while *Bacillus* sp. 6M2A had the highest glutamic acid accumulation at 1993.02 mol. l⁻¹. The strains of this study have demonstrated appropriateness for use as a cocktail culture to accelerate the fermentation process and enhance the flavor profile of fish sauces. Halotolerant nature of the strains and enzymes supports their potentials for wider use in the food industry.

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1. Introduction

Budu is a Malaysian traditional fish sauce made from anchovies fermented with salt for 6–12 m and officially recognized as a national culinary heritage. Flavor is addressed as the primary quality attribute of budu. Function prediction of budu microbial communities based on meta-genomic composition has demonstrated increased protein

and lipid breakdown pathways during fermentation and these pathways are known to play critical roles in formation of flavor [1]. Through spontaneous fermentation of high-salt fish sauce, a wide range of taste and aroma-active compounds belonging to aldehydes, acids, esters, alcohols and pyrazines are gradually formed, including 2-methyl-



butanal, 3-methylbutanoic acid, methyl 2-ethyldecanoate, 1-octen-3-ol and 2,6-dimethylpyrazine [1]. This is achieved through the combined activities of fish endogenous enzymes and metabolic activities of halophilic and halotolerant microbes [2]. Additionally, microbes are capable of producing microbial volatile organic compounds, which may stimulate production of secondary metabolites, especially during co-cultures [3]. Fatty acids, peptides and amino acids that are released through the process of lipid and protein hydrolyses serve as precursors for aroma compounds. They can be transformed into volatile organic compounds, including aldehydes, esters, ketones, alcohols, furans, hydrocarbons, lactones, pyrazines, amines, phenols, indoles, acids and sulfur-containing compounds [4]. The medium and short-chain fatty acid esters are important volatile organic compounds that can impart pleasant fruity flavors and aromas [5] and their presence in fish sauce can cover unpleasant smells and off flavors contributed by protein-derived compounds such as dimethyl trisulfide and trimethylamines. Microorganisms involved in fish sauce fermentation may facilitate glutamic acid accumulation by converting ketoglutaric acid into L-glutamic acid under the function of glutamate synthetase or glutamic acid dehydrogenase, as well as hydrolysis of proteins into umami amino acids and peptides [6]. Figure 1 shows schematic functions of protease, lipase and esterase as well as formation of glutamic acid in fermentation of fish sauces.

At the onset of fermentation, fermentation-assisting bacteria are not the prevailing microorganisms. They need

longer times to adapt to high salinity, establish dominance in the microbial community and produce flavor compounds [7]. Consequently, a prolonged fermentation time is needed to produce rich, flavorful fish sauces. The extended fermentation time can lead to an increase in overall production time, which may not be desirable for the producers who try to meet market demand and generate revenue quickly. In response to this, Malaysian fish-sauce producers terminate their fermentation process after only three months even though the flavor has not fully developed. Accelerating the fermentation process is therefore important for the production efficiency and profitability of fish sauce industries.

Use of starting cultures has been suggested as an effective method to enhance proteolysis during fish-sauce fermentation [8], while improving and maintaining the consistent quality of the fermented fish [9]. Addition of *Marinococcus halotolerans* SPQ isolated from salt crystals showed increased contents of aspartic and glutamic acids with several aroma compounds belonged to alcohol group [10]. Use of protease-producing *Tetragenococcus halophilus* has decreased fermentation time to six months and improved quantity of desirable amino acids [11]. When selecting starter cultures for fish-sauce fermentation, ease of cultivation is primarily important. It is essential for the strains to possess pro-technological metabolic characteristics such as the ability to grow and produce high-activity hydrolases that are active at high salt concentrations (20–30% NaCl).

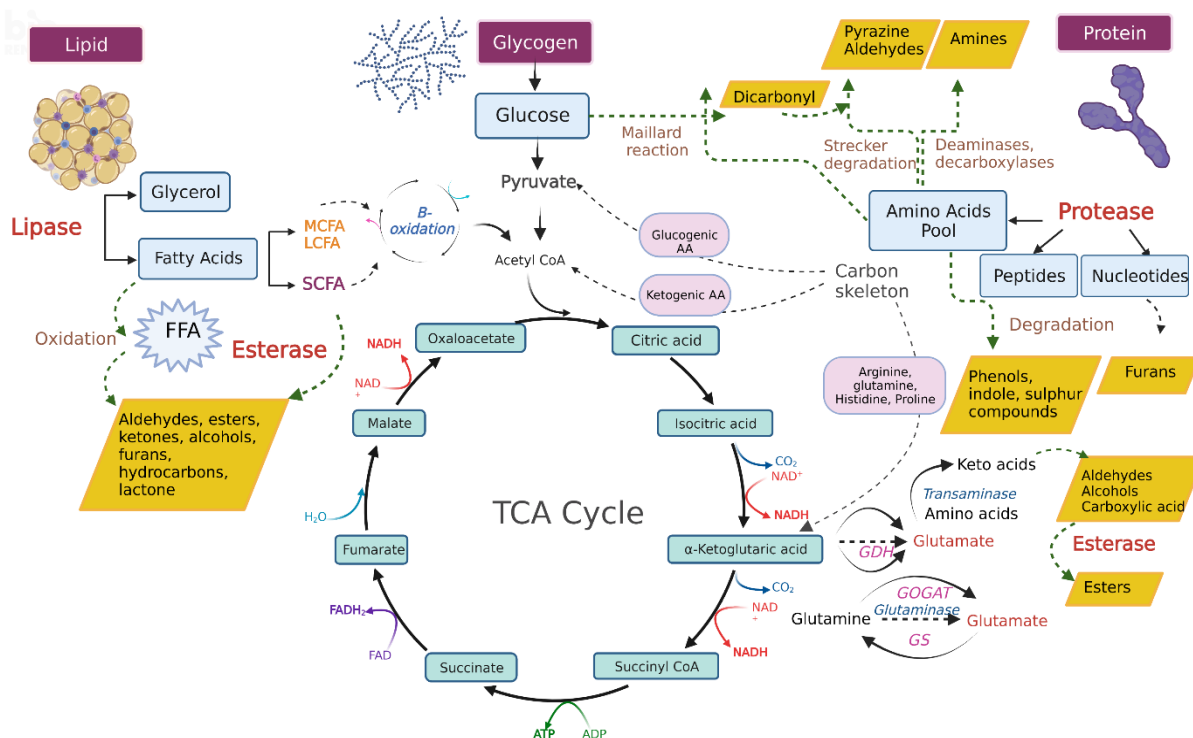


Figure 1. Summarized roles of protease, lipase, esterase and glutamic acid production activity in formation of taste- and aroma-active compounds in fish sauce fermentation (created with BioRender.com)

Therefore, a candidate derived from fish sauce is an excellent choice due to its adaptability, resistance to osmotic stress and ability to thrive in high salt environments. This enables them to grow more efficiently and fulfil their roles within the microbial community.

Fermentation of fish, which naturally contains high protein and low sugar contents, differs significantly from conventional carbohydrate fermentation. Rather than targeting sugars, this process focuses majorly on fish protein and lipid components [12]. Therefore, proteolytic and lipolytic activities are two key characteristics that should be addressed when selecting starter candidates for fish fermentation. Release of proteases and lipases by halophilic *Staphylococcus simulans* PMRS35 and *Haloarcula* sp. isolated from salted and fermented fish [13,14] as well as *M. halotolerans* SPQ and *Chromohalobacter* sp. from salt environments [10,15] has been documented in several investigations. It is suggested that fish sauce contains a diverse enzymes-producing microbiota that contribute to the formation of taste-active compounds, making it an ideal source for the selection of starter cultures. Nevertheless, a complete assessment of various strains from budu has not been carried out, specifically for assessing production of multiple enzymes that are likely to improve budu flavor. The aim of the current study was to isolate, identify and assess bacterial cultures from budu, displaying proteolytic, lipolytic, esterolytic and glutamic-acid accumulating activities. This study provides a novel contribution to the existing literature as it assessed the bacterial enzymes involved in the flavor development of Malaysian fish sauce, an area with limited attentions. Halotolerant strains thoroughly assessed for their major abilities to degrade fish proteins and lipids make promises for potential uses in the food industry. They can provide a viable solution for improving production and fermentation of high-salt fermented foods, particularly fish sauces.

2. Materials and Methods

2.1. Isolation of halotolerant proteolytic bacteria

Fish-sauce samples from 3, 6 and 12-m fermentation tanks were used for bacterial isolation. A modified isolation approach [8] was used to concurrently identify halotolerant proteolytic bacteria during first screening. To increase the likelihood of collecting indigenous proteolytic fish-sauce origin strains, media types and salt concentrations were modified. Twenty-five grams of fish sauce were diluted with 225 ml of sterile 0.85% (w/v) NaCl and stirred for 1 min. To recover further halophilic species, three decimal dilutions of the samples were plated on four media of marine agar (MA) with 20% NaCl for bacteria tolerant to 20% NaCl; De Man, Rogosa and Sharpe agar (MRSA) with 20% NaCl for 20% NaCl-tolerant lactic acid bacteria (LAB); mannitol salt agar (MSA) with 10% NaCl for salt-

tolerant Staphylococci; and nutrient agar (NA) with 30% NaCl for bacteria tolerant to 30% NaCl. Each medium received 1% of skim milk for proteolytic activity screening. The agar medium was incubated at 30 °C for 3–14 d until colonies formed. Four-quadrant streaking pattern was used to pick and streak clear zone-forming bacterial colonies with diverse morphologies onto newly prepared agar plates for purification.

2.2. Screening of protease and lipase/esterase activities using agar plate assay

Prior to the screening, one loop of every single isolate was transferred to marine broth (MB) containing 20% NaCl and incubated at 30 °C for 3 d, except for colonies recovered from MSA that were grown in nutrient broth supplemented with 10% NaCl. Cells were collected by centrifugation at 4000 rpm for 10 min at 4 °C following a 3-d incubation time. To prepare pellets for resuspension, pellets were first rinsed twice with 10 ml of pH-7.4 phosphate buffered saline (PBS). For each strain, absorbance of the suspended cells at 600 nm was adjusted to 0.85 before well-diffusion method was used. For the screening of bacteria with proteolytic activity, aliquots of the bacterial suspension (50 µl) were added to a 6-mm well on agar media consisted of NA supplemented with 10% NaCl and 1% skim milk for MSA-derived strains and MA supplemented with 1% skim milk for the rest of strains. Proteolytic indices were assessed after incubating plates at 30 °C for 2 d [9]. Agar spot techniques in three various media of phenol red, tributyrin and Tween 80 agar were used to screen bacteria that produced lipase or esterase. Each medium was prepared according to [16]. Five microliters of resuspended cells were dropped onto the surface of the phenol red, tributyrin and Tween 80 agar and allowed to absorb. Inoculated plates were incubated at 30 °C for 3–14 d until visible signs of lipolysis activity were observed. The color shift of phenol-red agar from red to orange indicated lipase activity, which was caused by a slight pH drop induced by fatty acid releases. A clear zone on tributyrin agar indicated the presence of extracellular esterase. The FFAs were precipitated by calcium (giving a white zone) around colonies showing lipase and esterase activity on Tween 80 agar. Isolates demonstrating a high proteolytic index and/or indicating lipolytic activity were selected for further growth and protease activity assessment using liquid assay.

2.3. Growth assessment of the strains

One loop of each selected strain was transferred to MB media and incubated at 30 °C for 48 h. After incubation, cells were centrifuged at 4000 rpm for 10 min at 4 °C. Collected pellets were washed and resuspended in PBS with an OD₆₀₀ adjusted to 1.1–1.2. A 1-ml volume of the cell suspension was transferred to 40 ml of MB using 125-ml amber bottles. The mixture was incubated at 30 °C with 120-



rpm agitation speed. Growth assessment was carried out by measuring aliquots of the growth media at 600 nm using Biomate 3 UV-vis spectrophotometer (Thermo-Fisher Scientific, Massachusetts, USA) at specified intervals for 216 h. Sterile MB medium was used as blank for each reading.

2.4. Extraction of the bacterial crude enzymes

Cell-free supernatant (CFS) was prepared for the enzyme assays by centrifuging aliquots of the harvested growth media at $11,000 \times g$ for 10 min at 4 °C.

2.5. Assessment of protease activity

Protease activity of the isolates was determined based on the Cupp-Enyard and Aldrich method [17], where casein was used as substrate. Casein was diluted to 6.5 mg.ml^{-1} in 50 mM potassium phosphate buffer (pH 7.5). Temperature of the solution gradually increased to 80–85 °C with gentle stirring for nearly 10 min until a homogeneous dispersion was achieved. Casein solution was set at 37 °C prior to use. Moreover, CFS was added to 5 ml of casein solution and incubated at 37 °C for 10 min. Every tube was then treated with 5-ml solution of 110 mM trichloroacetic acid (TCA) following incubation to terminate the reaction. To verify that each tube had an identical end volume and account for the enzyme absorbance value, an equal quantity of the enzyme solution was added to the blank. Solutions were incubated at 37 °C for 30 min and then centrifuged at 4000 rpm for 5 min to remove insoluble substances. For colorimetric determination of tyrosine, 400 µl of the supernatant were immediately added with 1 ml of sodium carbonate and 0.2 ml of Folin reagent. The mixture was mixed gently and permitted to stabilize at 37 °C for 30 min. Absorbance measurement was carried out at 660 nm using spectrophotometer. Tyrosine concentration was determined using standard tyrosine curve. Bacterial protease unit (U) was defined as the quantity of enzyme that produced the equivalent of 1 µmol. L⁻¹ of L-tyrosine per min in pH 7.5 and 37 °C. Isolates with significant protease activity were subjected to further analyses. The protease activity was determined using the Eq. 1

$$\text{Protease activity (U)} = \frac{\text{tyrosine conc.} \left(\frac{\mu\text{mol}}{\text{l}} \right) \times \text{total assay volume (ml)}}{\text{ml CFS} \times \text{assay time} \times \text{ml used in colorimetric}} \quad \text{Eq.1}$$

2.6. Effects of time, temperature and NaCl concentration on protease and lipase activities

Using MB media, the selected strains were assessed for their protease and lipase activities under various temperatures (30 and 35 °C) and NaCl concentrations (20, 25 and 30%). In this study, a temperature range of 30–35 °C was selected as it represented the average temperature used in the industry. Salt concentration varied 20–30% during budu 12-m fermentation; therefore, this salt range was used to assess its effects on the strains' enzyme activity. Protease

assay was carried out using the method described in Section 2.5. To assess lipolytic activity, titration method [18] was used with emulsion of fish oil and polyvinyl alcohol at a ratio of 1:3 as the substrate for the assay. Briefly, 2.5 ml of phosphate buffer (pH 4.8) were added to 2.5 ml of the substrate, followed by adding 0.5 ml of CFS. A 10-min enzymatic reaction was carried out at 37 °C. Blank was prepared without addition of CFS. A final volume of 7.5 ml of 95% ethanol was added to terminate the reaction. Phenolphthalein indicator was used to titrate the released fatty acids against 0.1 M NaOH. The quantity of lipase that might release 1 µmol of fatty acids was measured in units of lipase activity. The mole of fatty acids was equal to the mole of the titrant and 1 mol NaOH was equal to $10^6 \times \mu\text{mol}$ fatty acid equivalent. Lipase activity was determined using the Eqs. 2 and 3:

$$\text{mol NaOH} = 0.1 \frac{\text{mol}}{\text{l}} \times \frac{(\text{ml titration sample} - \text{ml titration blank})}{1000 \text{ ml/l}} \quad \text{Eq. 2}$$

$$\text{Lipolytic activity (U)} = \frac{(\mu\text{mol fatty acids eq.} \times \text{total assay volume (ml)})}{\text{ml CFS} \times \text{assay time} \times \text{ml used in NaOH titration}} \quad \text{Eq. 3}$$

2.7. Esterase activity

Colorimetric method was used to determine the esterase activity using modified method [19, 20]. Prior to esterase assessment, an overnight culture (24 h) of each strain was grown in modified Gibbon's media containing 20% of NaCl, 2.5% of fish oil, 0.1% of MgSO₄·7H₂O, 0.3% of Na₃C₆H₅O, 0.2% of KCl, 0.75% of tryptone and 0.1% of yeast extract for 3 d at 30 °C and 120 rpm. The CFS was used for the analysis. A mixture of 14 mM p-nitrophenyl butyrate in acetonitrile, 66.67 mM of Tris HCl (pH 8) and 3M of NaCl at a ratio of 1:8:1 was prepared as the substrate for the esterase activity assay. A volume of 20 µl of CFS and 200 µl of substrate were transferred into 96-well microplates and gently agitated. Double-distilled water was used as blank instead of CFS. Absorbance reading was carried out at 410 nm every 30 s at 37 °C over 5 min to measure the p-nitrophenol (p-NP) release using microplate reader (Powerwave X 340 microplate scanning spectrophotometer, BIO-TEK Instruments, Vermont, USA). Extinction coefficient (ε) for p-NP of this assay was 8222.1 M⁻¹ cm⁻¹, which was derived from the slope of the p-NP standard curve under similar detection conditions. For one unit (U) of esterase activity, enzyme quantity needed to release 1 µM of p-NP per minute was calculated based on Eq. 4 and Beer-Lambert Law as follows:

$$\text{Esterase activity} \left(\frac{\text{U}}{\text{ml}} \right) = \frac{10^6 \times \Delta\text{Abs} \times \text{volume of assay (ml)}}{\epsilon \times \Delta t (\text{min}) \times \text{volume of enzyme (ml)}} \quad \text{Eq. 4}$$

Where, ΔAbs showed changes in absorbance over time; ε denoted the molar extinction coefficient in M⁻¹ cm⁻¹;



volume of assay represented the total volume of reaction mixture (ml); and Δt represented the incubation time (min).

2.8. Glutamic acid assessment

Prior to assessment, the overnight culture of each strain was inoculated (1% v/v) into the glutamic acid production media (pH 7). This medium consisted of 5% of glucose, 0.8% of urea, 0.0002% of biotin, 0.1% of K_2HPO_4 , 0.25% of $MgSO_4 \cdot 7H_2O$, 0.01% of $MnSO_4 \cdot 7H_2O$ and 0.16% of $CaCO_3$. Incubation was carried out at 30 °C for 7 d at 120 rpm. The crude glutamic acid source was the CFS that separated following centrifugation at 10,000 rpm for 10 min at 4 °C [21]. Glutamic acid was determined using glutamic acid colorimetric assay kit (Elabscience Biotechnology, Texas, USA).

2.9. Genome-based identification of the isolates

Extraction of the bacterial DNA was carried out using Nucleospin microbial DNA extraction kit (Machery-Nagel, Duren, Germany). One loop of a single bacterial colony was pelleted and transferred to a microcentrifuge tube containing 40–400 μm of glass beads, added with buffer and proteinase and processed with FastPrep-24 system (MP Biomedicals, Ohio, USA). Lysed cells were centrifuged at 11,000 $\times$ g for 30 s and DNA in the supernatant was purified using microbial DNA column based on the manufacturer's protocol. Genome-based identification of the strains was carried out using Patriot Biotech, Selangor, Malaysia, based on the manufacturer's standard protocol. Primers of 27F (TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG) and 1492R (ACTTGCCTGTCGCTCTATCTTCTACGGYTACCTTGTTACGACTT) with a Nanopore partial adapter on the 5' end were used to amplify 16S rRNA full-length sequence of the strains. The PCR was carried out using WizBio HotStart 2 $\times$ mastermix (Wizbiosolutions, Gyeonggi-do, Korea) with the following conditions of an initial denaturation step at 95 °C for 3 min; followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 20 s and extension at 72 °C for 120 s. The PCR products were visualized on agarose gels and purified using solid-phase reversible immobilization beads. Nanopore Flongle flow cell (Oxford Nanopore Technologies, Oxford, UK) was used for the 24-h sequencing process. Sequences were compared to known sequences using BLAST online tool of the National Centre for Biotechnology (NCBI) website to verify the homology. The neighbor-joining trees were then constructed using MEGA 11 software. The rRNA sequence data of the selected strains were submitted to GenBank database.

2.10. Statistical analysis

Minitab 19 statistical package (Minitab, Pennsylvania, USA) was used for the analysis. Results of the ANOVA analysis was reported statistically significant when $p < 0.05$. One-way ANOVA was carried out on variables to detect

significant differences between the samples. Four-way ANOVA was used to assess effects of the multiple factors (time, temperature, NaCl concentration and strain) on the protease and lipase activities. To find out which groups of the samples were distinct from others, post hoc Tukey's honest significant difference (HSD) test was carried out.

2.11. Ethical considerations

No experiments involving humans or animals were carried out in this study.

3. Results and Discussion

3.1. Initial screening and isolation of proteolytic and lipolytic halotolerant bacteria

For selecting bacteria for budu starter cultures, strains were initially assessed based on their ability to persist high-salt concentrations associated with budu fermentation. A total of 50 bacterial isolates were capable of growing on isolation media supplemented with 10–30% of NaCl (w/v) (data not shown). Twenty-nine isolates were originated from 12-m samples, eight from 6-m samples and 13 from 3-m samples. Only five isolates were recovered from NA containing 30% of NaCl, while 40 isolates were recovered from MA (20% NaCl). No colonies were observed in the MRSA containing 20% of NaCl, whereas, a few staphylococci were detected on MSA with 10% of NaCl. Decreased number of strains recovered from budu when grown on high-salt media could be due to the inactivation of key enzymes for microbial metabolism and growth. Additionally, the osmotic stress and metabolic adaptation forced bacteria to allocate their energy for adaptation rather than multiplication. Under further severe conditions, bacteria might enter viable but nonculturable (VBNC) states [22]. In a previous study, LAB and halophilic bacteria were isolated from Vietnamese fish sauces using salt concentrations ranging 5–18 and 18–25%, respectively. No growth of colonies was detected in high-salt media (approximately 29% NaCl) [10]. In the present study, strains capable of growing at 30% NaCl could be recovered; however, a longer incubation time of 14 d was needed for the colonies to become visible in the media, in contrast to the growth on MA that occurred within 72 h.

Presence of LAB has been reported in Thai and Japanese fish sauces [11, 23]; however, none of this group was recovered from budu when plated on MRSA containing 20% of NaCl. Absence of LAB growth in this media was because a majority of LAB grew best at a NaCl concentration of less than 10%, except for a few species in *Tetragenococcus* genus. All the 50 salt-tolerant bacteria from the initial screening could produce zones of hydrolysis surrounding colonies to various degrees, ranging 1.3–4.1 cm when grown on skim milk agar. However, only a few strains demonstrated lipase/esterase activities on phenol red,



tributylin and Tween 80 agar (data not shown). Figure 2 shows growth of colonies on various solid media during the initial screening of protease and lipase activities.

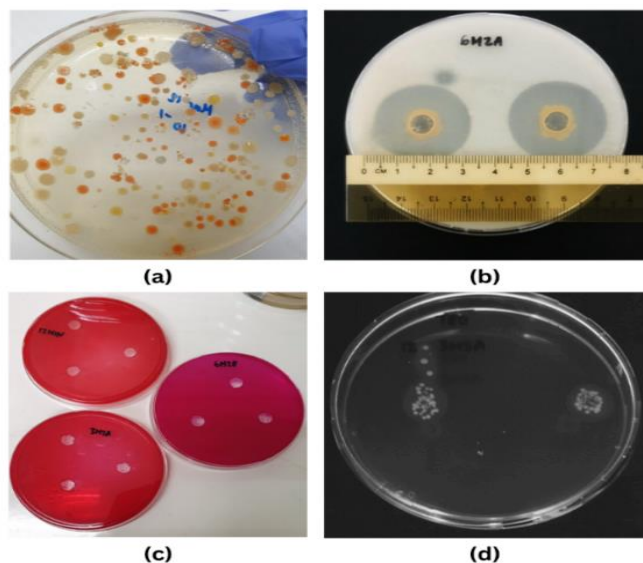


Figure 2. Growth of colonies on different solid media: MA (20% NaCl) supplemented with 1% skim milk (a); formation of a proteolysis zone by isolate 6M2A (b); change in color of phenol red agar to orange-red, indicating the release of fatty acids by isolates 3M3A and 12M1N (c); formation of a white halo surrounding colonies indicating lipolytic activity by isolate 3M3A (d)

Twelve out of 50 isolates (Table 1), which selected based on the proteolytic index and qualitative lipase/esterase activity, were assessed for growth curve and protease activity using casein-based protease activity assay.

3.2. Growth assessment and screening of the protease activity using liquid assay

In Figure 3, growth curve and protease activity of 12 different strains are shown. Strains entered the lag phase from 0–6 h and the log phase from 6–24 h. From 24 h, stationary phase was occurred.

A combined timeline of the growth curve and protease activity indicated that protease was highly produced during the early to mid-stationary phases. Findings were similar to those from previous reports [24]; in which, *Bacillus subtilis* and *B. siamensis* demonstrated peaked protease activities during the stationary phase of bacterial growth. The logarithmic growth phase was observed within 12–24 h for all the strains. This phase was characterized by rapid division and growth of bacteria, which needed nutrients and energy for protein synthesis and cell replication. This led to upregulation of protease synthesis to assist in protein degradation and amino acids production for cellular metabolism. Stress caused by carbon and nitrogen depletion might contribute to the high production of proteolytic enzymes during the stationary phase [25].

Table 1. Twelve isolates passing the initial screening for protease, lipase and esterase activities

Strains	Isolation source (batch fermentation) (months)	Isolation medium	Dilution	Colony morphology (color, elevation, texture, shape, margin)	Proteolytic Index	Growth on Lipase/Esterase Media		
						Phenol red	Tributylin	Tween 80
3M2G	3	Marine Agar	10 ²	yellow, umbonate shiny, irregular, smooth	2.16±0.48	-	-	-
3M3A	3		10 ³	milky white, umbonate, matte, irregular, undulate	2.17±0.08	+	+	+
6M1C	6		10 ¹	cream, convex, shiny, irregular, lobate	2.35±0.63	-	+	-
6M2A	6		10 ²	Yellow, convex, shiny, irregular, undulate	1.66±0.22	+	+	+
6M2C	6		10 ²	milky white, wrinkled, brittle, irregular, lobate	1.46±0.06	+	+	+
12M1B	12		10 ¹	pink, convex, shiny, round, curled	1.56±0.01	-	-	-
12M1C	12		10 ¹	light yellow, convex, shiny, round, smooth	1.54±0.06	+	+	+
12M1F	12		10 ¹	cream, raised, shiny, round, smooth	1.65±0.16	-	+	+
12M1N	12		10 ¹	cream, flat, brittle/matte, round, undulate	1.80±0.38	-	+	+
12M1J	12		10 ¹	cream, flat, brittle/matte, round, smooth	1.83±0.11	+	+	+
12N1B	12	Nutrient Agar	10 ¹	milky white, flat, matte, irregular, lobate	2.10±0.14	-	+	+
12N3A	12		10 ³	milky white umbonate, wrinkled/matte, filamentous, lobate	1.97±0.16	-	-	+

Significant differences ($p < 0.05$) across strains were denoted by superscripts with distinct letters in the column.



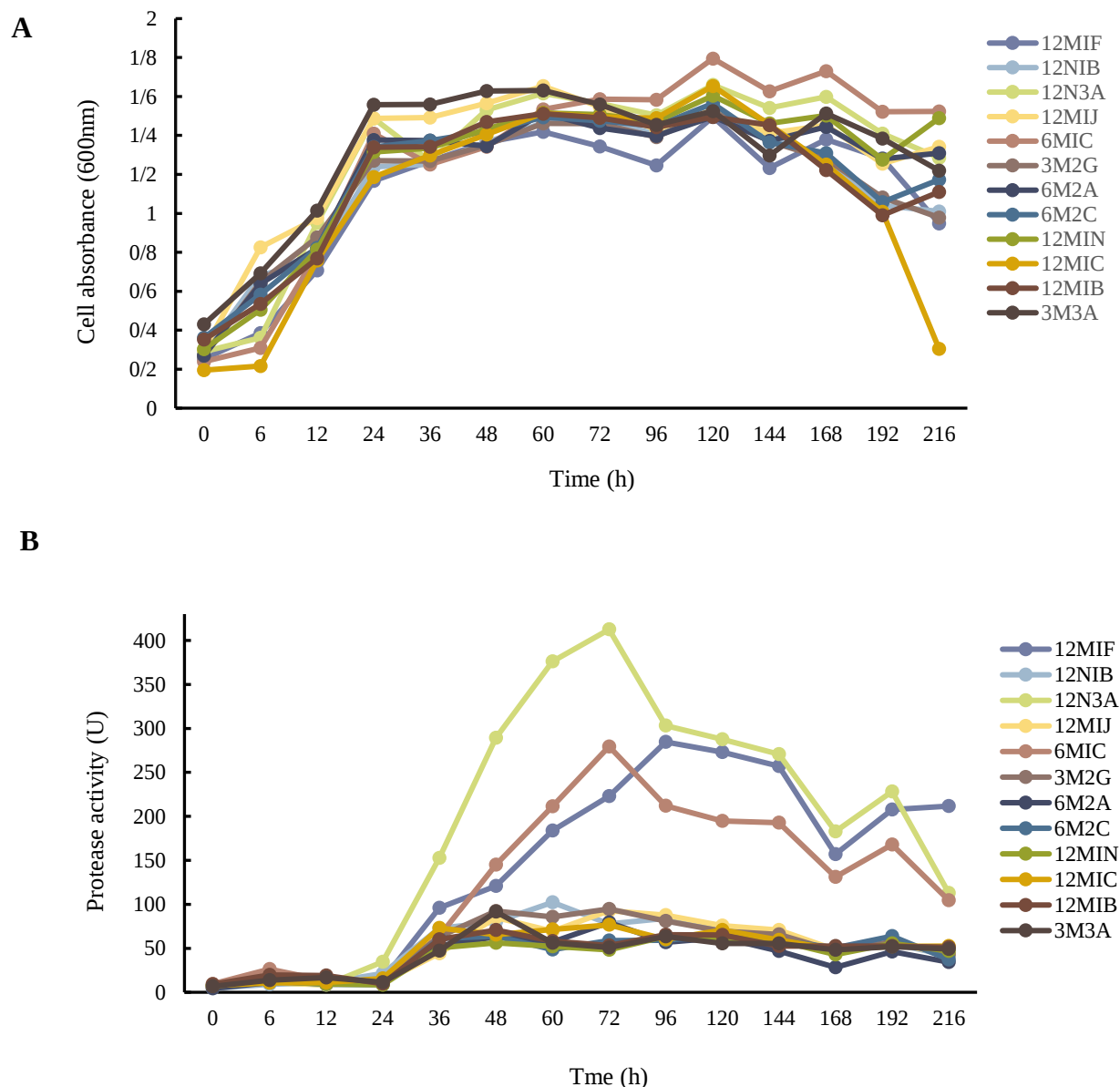


Figure 3. Growth kinetics (a) and protease activity (b) of strains isolated from *budu*

Out of the 12 strains, 12N3A demonstrated the highest protease activity reaching 412.5 U within 72 h, which corresponded to the mid-stationary phase. This *budu* strain proteolytic activity was higher than that of *B. subtilis* isolated from natto, a fermented soybean, which ranged 122.64–280.90 U after 24 h of incubation [26]. The six strains with the highest proteolytic activity were then assessed for their lipolytic and proteolytic activities under various growth conditions.

3.3. Effects of incubation time, temperature and salt concentration on the strain protease activity

Figure 4 shows dynamics of protease activity of six selected strains of 3M2G, 3M3A, 6M1C, 6M2A, 12M1F and 12N3A.

Figure 4 illustrates that 6M1C, 6M2A and 12M1F achieved their highest protease activity at 96 h with the values of 161.82 U, 164.78U and 144.99 U, respectively. In contrast, the highest protease activities of 3M2G, 3M3A and 12N3A strains were reached after 48 h with the respective values of 158.27 U, 140.51 U and 177.21. Significant effects of incubation time, temperature, NaCl, strain and their interactions on the protease activity ($p < 0.05$) were verified based on Tukey's HSD post hoc test that was carried out following four-way ANOVA. With an average value of 102.59 U, protease activity reached its highest point after 96 h of incubation. For temperature, the highest protease activity of all strains for all the factors was reached at 30 °C. Temperature fluctuation is one of the most common



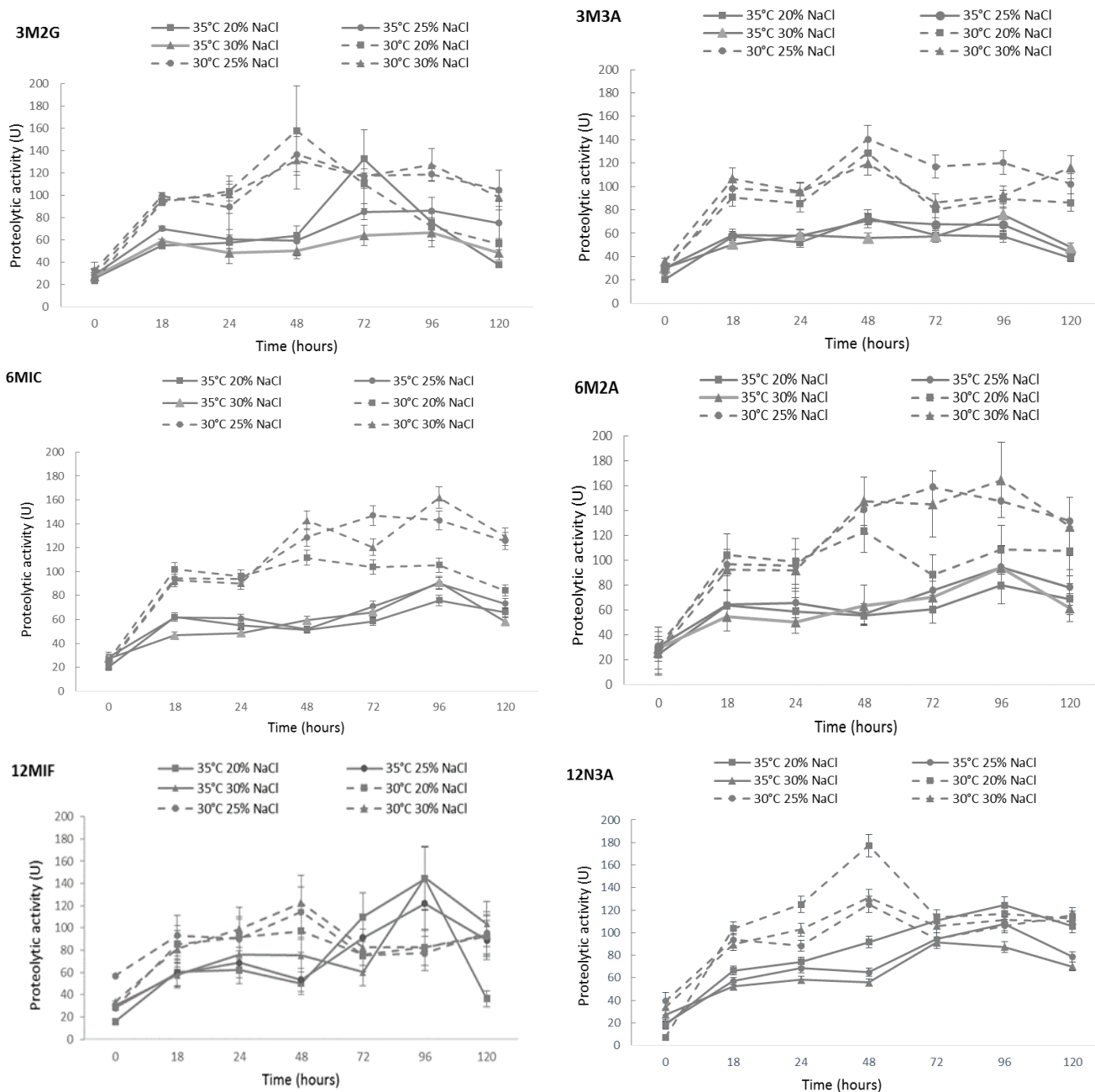


Figure 4. Protease activities of six budu strains as functions of time, temperature and NaCl concentration

environmental conditions during budu fermentation in Malaysia, which is generally carried out in open-air environments. The internal fermentation temperatures for budu range 30–35 °C while in Korean fish-sauce production, 30 °C is an ideal temperature for fermentation [27]. Budu isolates are typically adapted to grow in high-salt environments as they have developed mechanisms to tolerate excessive NaCl concentrations. Salt concentrations of 25% were optimum for enhanced protease activities of budu isolates, followed by 30% of NaCl. The NaCl concentrations up to 20% increased the *Virgibacillus* sp. SK37 extracellular protease activity [28]. The two strains of

12N3A and 6M2A produced the highest levels of proteases overall, indicating that protease activities were strain-specific. Adaptive variations in the environment, genetic variations of the protease-producing genes and differences in gene expression regulation might explain differences in the ability to produce proteases between the isolates [29].

3.4. Effects of incubation time, temperature and salt concentration on the strain lipase activity

Figure 5 illustrates the strain lipolytic activities at various temperatures and NaCl concentrations over 120-h of the growth time.



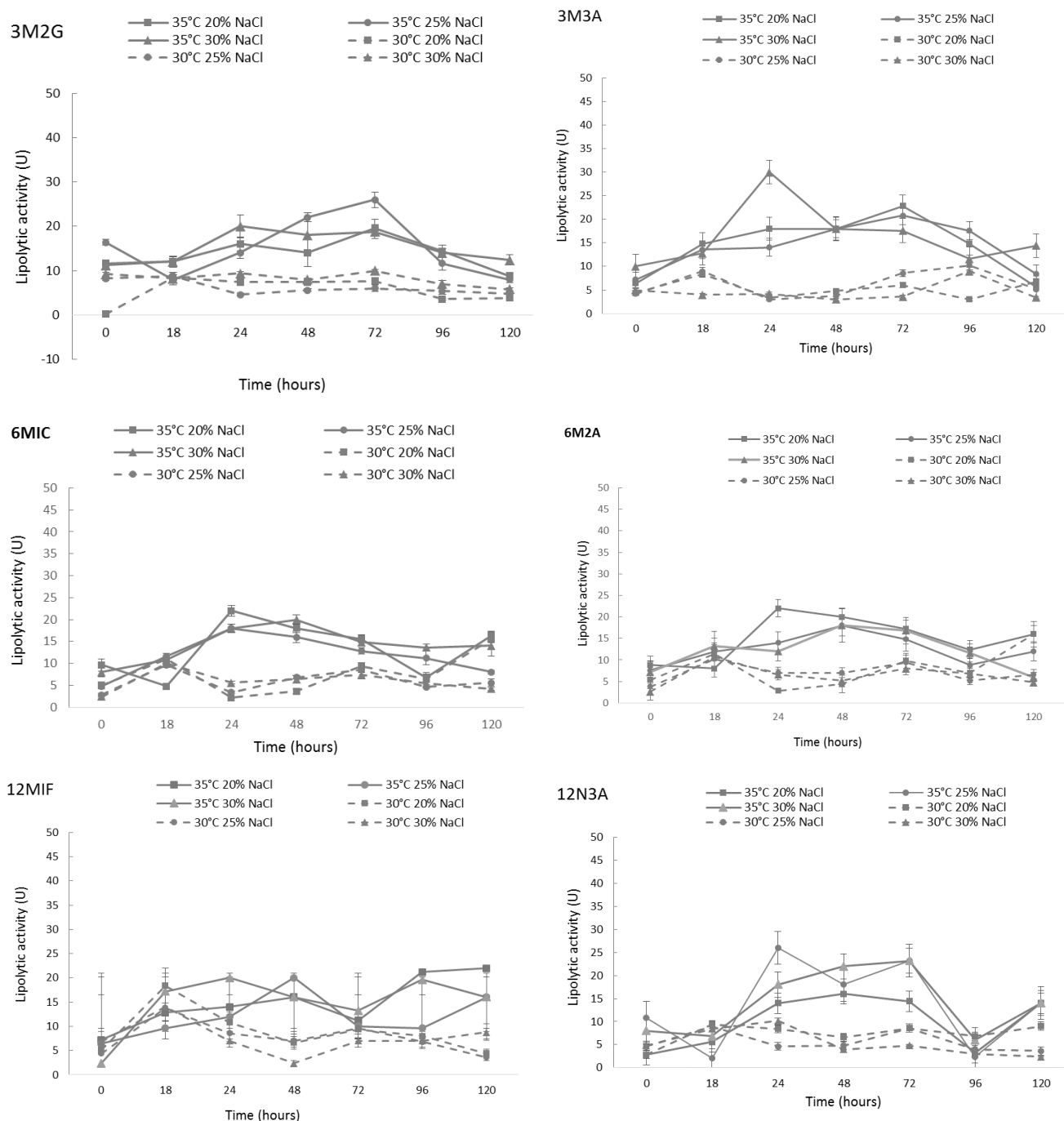


Figure 5. Lipase activities of six budu strains as functions of temperature and salt concentration

A four-factor ANOVA (time, temperature, NaCl concentration and strain) indicated significant positive correlations between all factors and their interactions and protease activity ($p < 0.05$). The greatest lipase activity was seen after 72 h of growth, as shown by Tukey's HSD pairwise comparison with time as a factor with an overall mean value of 12.58 U. This activity did not differ significantly from that of 24 h (11.92 U), which was the second highest lipase activity. The high lipolytic activity in the early stage of stationary phase (24–72 h) was possibly correlated with the glucose depletion in the growth media

following the microbial rapid growth during the log phase, which resorted use of carbon source from glucose to lipids; hence, activating the lipase activity. Tukey's HSD pairwise comparison showed a generally higher lipase activity at 35 °C than 30 °C. Findings of this study were similar to the findings of a previous study, which reported significant decreases in lipase activity, when temperature increased from 25 to 40 °C [30]. Lipolytic activity was the highest at 30% NaCl and no significant differences were observed between this concentration and 20% NaCl. Despite secretion by similar strains, lipase and protease activities



Table 2. Esterase activities and glutamic acid production capacities of budu strains

Strains	Esterase activity (Unit)	Glutamic acid production (μ mol.L ⁻¹)
3M2G	118.69±18.65	1106.98±166.22 ^{ab}
3M3A	155.48±15.27	902.33±374.9 ^b
6M1C	97.47±35.41	1325.58±210.49 ^{ab}
6M2A	124.80±44.87	1993.02±36.18 ^a
12M1F	152.42±36.62	1155.81±243.75 ^{ab}
12N3A	104.45±26.08	1367.44±256.53 ^{ab}

Significant differences ($p < 0.05$) across strains were denoted by superscripts with distinct letters in the column.

could have various optimum temperatures and salt concentrations, as observed in this study. Differences were possibly caused by the fact that enzymes are proteins and each protein has its own structure, allowing it to react differently to external factors such as NaCl and temperature. Overall, 12M1F and 3M2G strains presented the highest crude lipase activities with the values of 10.91 and 10.67 U, respectively. A *B. subtilis* FS2 strain of Vietnamese fish-sauce origin was reported to secrete enzymes with lipase A activity when assessed on 0.1% tributyrin agar [31].

3.5. Esterase activity

Table 2 shows that esterase activity of the strains from budu ranged 97.47–155.48 U with 3M3A strain demonstrated the highest esterase activity (155.48 U), followed by 12M1F and 6M2A strains. Differences in the activity were strain specific and could be affected by genetic variations and gene expression of each strain. The esterase activity of halotolerant *Salimicrobium* sp. was initially detected during the mid-exponential growth phase (16–28 h) and reached to its peak at 24 U.mL⁻¹ in the stationary phase (30–48 h) [32]. The pattern closely resembled activities of the protease and lipase in the current study. *Bacillus licheniformis* was reported to have its maximum esterase activity after 24 h of incubation [33]. A maximum specific esterase activity of 21.23 U.mg⁻¹ was seen for *Bacillus* sp. JR3, using p-nitrophenyl butyrate as substrate [34]. Similar to previous findings [35], all the six halotolerant budu isolates displayed combined hydrolytic activities as they were capable of producing proteases, lipases and esterases.

3.6. Glutamic-acid production activity

Glutamic acid, which contributes to fish-sauce umami flavor, can be produced via bacterial metabolism and protein degradation. Glutamic-acid accumulation abilities of the strains in the specific growth media are present in Table 2. Glutamic acid concentrations produced by the six strains ranged 902.33–1993.02 μ mol. l⁻¹. Using glucose as fermentation substrate, strains of 6M2A, 12N3A, 6M1C, 12M1F and 3M2G produced higher glutamic acid concentrations than that *Lactobacillus plantarum* strain MNZ isolated from fermented foods did, whose activity was 1.032 mmol.l⁻¹ [36]. Technically, *gdh* gene has been identified in *Bacillus* sp., which is responsible for producing

glutamic acid [37]. Glutamic acid decarboxylase in *B. megaterium* (BmGAD) are capable of accumulating L-glutamic acid at pH 5 with a specific activity of approximately 150 U.mg⁻¹ [38].

3.7. Strain identification

The 3M2G, 6M1C, 6M2A, 12M1F and 12N3A strains showed the highest homology of more than 99% with *B. haikouensis*, *Bacillus* sp. V3, *Bacillus* sp. V3, *B. haikouensis* and *B. licheniformis*, respectively, indicating that species-level identification was achieved. In contrast, 3M3A strain demonstrated 97.23% homology with *B. licheniformis*, suggesting that this strain belonged to *Bacillus* genus; thus, *Bacillus* sp. 3M3A was proposed for this strain. Sequencing data for each strain were deposited in GenBank under the following reference nos. of OQ642133, OQ642134, OQ642135, OQ642136, OQ642137 and OQ642138. Complete 16S rRNA gene sequences of all isolates were phylogenetically analyzed. Results (Figure 6) demonstrated that 12N3A and 3M3A isolates shared similarities with a variety of *B. licheniformis* strains. For genetic relatedness, 6M2A and 6M1C strains were closely linked to *B. haikouensis*, *Rosellomorea aquimaris* and *R. vietnamensis*. *Bacillus haikouensis* is an anaerobic halotolerant bacterium that was initially discovered from paddy soil and has the capacity to grow on up to 17% NaCl [39].

The *R. vietnamensis*, which has been synonymized with *B. vietnamensis*, is a mesophilic aerobic species isolated from Vietnamese fish sauces that is capable of forming spores [40, 41]. Identified as *B. haikouensis*, 12M1F and 3M2G strains had close relationships with *R. vietnamensis*, sharing more than 99.8% of 16S rRNA gene sequences. This finding might indicate possible similarities in the characteristics of Malaysian and Vietnamese fish sauces. *Bacillus* has been addressed as one of most prevalent and beneficial microbes in fermented fish mostly due to its unique contribution during the fermentation process [42]. *Bacillus* strains isolated from fish sauces had amino oxidase activity, which could decrease biogenic amine accumulation during fish-sauce fermentation; thus, improving safety of the final products.



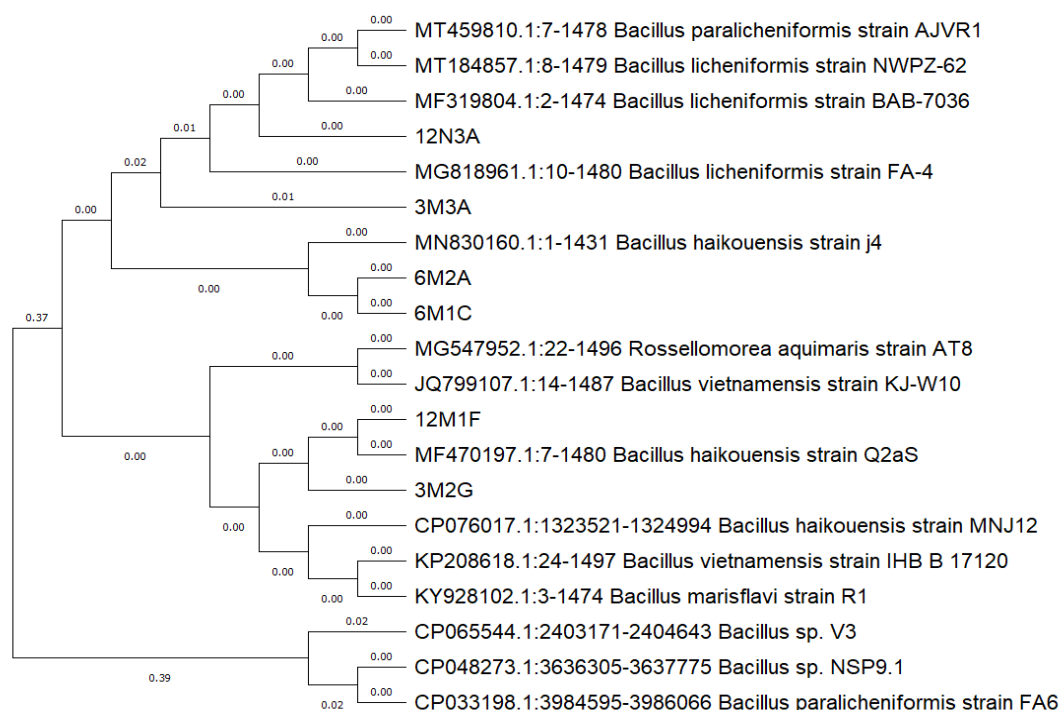


Figure 6. Maximum likelihood phylogenetic tree based on entire 16S rRNA gene sequences from the six strains isolated from budu and their relationships with other strains

Moreover, certain *Bacillus* strains derived from fermented foods have demonstrated probiotic activities in *in vitro* and *in vivo* experiments, which further expands their benefits in addition to their abilities to produce a variety of enzymes. Production of proteases, lipases, lipopeptides and extracellular polymers by probiotic *Bacillus* spp. enhances taste, nutritional value and safety of fermented foods [43]. Finding of this study have contributed to the body of knowledge in term of *Bacillus* strains possessing the ability to produce various enzymes. These strains can be used in the food industry, particularly for applications requiring enzymes and microorganisms that can persist extreme salinity. They may effectively be integrated with traditional fish fermentation practices to accelerate fermentation and enhance the flavor profile of fish sauces.

4. Conclusion

In this study, six *Bacillus* strains isolated from Malaysian fish sauces were isolated, demonstrating hydrolytic and glutamic acid-accumulating capacities. Proteases were generally higher at 30 °C and 25% NaCl and in the middle of the stationary phase with *B. licheniformis* 12N3A had the highest protease activity. In contrast, lipase activity was maximum at 35 °C and 30% NaCl and the onset of the stationary phase with *B. haikouensis* 12M1F. *Bacillus* sp. 3M3A and *Bacillus* sp. 6M2A demonstrated the highest esterase and glutamic-acid accumulating activities, respectively. Stability and resistance to NaCl of halotolerant strains and their enzymes are essential in a variety of scientific, industrial and environmental applications.

Furthermore, hydrolases produced by strains from various ecological environments may have unique characteristics and abilities, which can expand the range of enzymes available for uses in specific areas.

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6. Conflict of Interest

The authors declare no conflict of interest.

7. Authors Contributions

Conceptualization, S.D.L.; methodology, S.D.L., N.H.; software, S.D.L.; validation, N.H.; formal analysis, S.D.L.; investigation, S.D.L.; resources, N.H., A.S.M.H., S.M. and Y.S.S.; data curation, N.H.; writing—original draft preparation, S.D.L.; writing-review and editing, S.D.L., N.H., A.S.M.H. & Y.S.S.; visualization, S.D.L.; supervision, N.H., A.S.M.H., S.M. and Y.S.S.; project administration, S.D.L., N.H.; funding acquisition, S.D.L. and N.H.

8. Using Artificial Intelligent Chatbots

Artificial Intelligent chatbots have not been used in any section of the manuscript.



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چند سویه باسیلوس به دست آمده از سس های ماهی مالزیایی دارای توانایی پروتئولیتیک، لیپولیتیک، استروولیتیک و تولید گلوتامیک اسید

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واژگان کلیدی

- بودو (Budu)
- آنزیم ها
- تخمیر
- هالوتولرانس
- کشت آغازگر

نویسنده مسئول

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چکیده

سابقه و هدف: عطر و طعم سس ماهی ناشی از واکنش های متابولیکی پیچیده ای است که شامل باکتری ها و آنزیم های با توانایی تجزیه پروتئین ها و لیپیدها می باشد. زمان تخمیر طولانی تری برای ایجاد طعم شدید مورد نیاز است. هدف از این مطالعه، جداسازی باکتری های هالوتولرانت از سس ماهی تخمیر شده مالزیایی بود که فعالیت های پروتئولیتیک، لیپولیتیک، استروولیتیک و تجمع گلوتامیک اسید را نشان دادند. این باکتری ها ممکن است به طور بالقوه به عنوان کشت های آغازگر برای افزایش طعم ها در طی تخمیر سس ماهی مورد استفاده قرار گیرند.

مواد و روش ها: غربالگری اولیه فعالیت پروتئولیتیک روی سالین شیر بدون چربی اگر انجام شد. جدایه های با شاخص پروتئولیتیک بالا و فعالیت لیپاز/استراز مثبت بیشتر با استفاده از سنجش فعالیت پروتئاز مبتنی بر کازئین مورد ارزیابی قرار گرفتند. فعالیت های پروتئاز و لیپاز تحت شرایط گوناگون سویه ها، غلظت (۳۰-۲۰ % w/v) NaCl، دما (۳۵-۳۰ درجه سلسیوس) و زمان گرمخانه گذاری (۰-۱۲۰ ساعت) با استفاده از آنالیز واریانس چهار طرفه ارزیابی و تجزیه و تحلیل شد. p-نیتروفنیل بوتیرات و بررسی با روش رنگ سنجی به ترتیب برای تعیین فعالیت های تجمع استراز و گلوتامیک اسید استفاده شد.

یافته ها و نتیجه گیری: شش سویه جدا شده از budu فعالیت های هیدرولیتیکی را نشان دادند و به عنوان گونه های باسیلوس شناسایی شدند. فعالیت های پروتئولیتیک به طور متوسط در باسیلوس لیسنی فورمیس 12N3A در ۲۵٪ NaCl، ۳۰ درجه سلسیوس و ۹۶ ساعت گرمخانه گذاری بالاترین بود. بالاترین فعالیت لیپولیتیک باسیلوس هایکونسیس با 12M1F در ۳۰٪ NaCl، ۳۵ درجه سلسیوس و ۷۲ ساعت گرمخانه گذاری به دست آمد. باسیلوس گونه 3M3A بالاترین فعالیت استراز ۱۵۵.۴۸ U را نشان داد، در حالی که باسیلوس گونه 6M2A بالاترین تجمع گلوتامیک را در ۱۹۹۳/۰۲ mol. L⁻¹ داشت. سویه های این مطالعه برای استفاده به عنوان یک کشت کوکتل برای تسریع فرآیند تخمیر و افزایش طعم سس ماهی مناسب هستند. ماهیت هالوتولرانس سویه ها و آنزیم ها از پتانسیل آنها برای استفاده گسترده تر در صنایع غذایی حمایت می کند.

تعارض منافع: نویسندگان اعلام می کنند که هیچ نوع تعارض منافی مرتبط با انتشار این مقاله ندارند.