Current Microbiology
https://doi.org/10.1007/s00284-021-02481-1

Accumulation of Ectoines By Halophilic Bacteria Isolated from Fermented Shrimp Paste: An Adaptation Mechanism to Salinity, Temperature, and pH Stress

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Received: 23 September 2020 / Accepted: 26 March 2021

Abstract
Shrimp paste is a traditional fermented food produced by many Asian countries. Bacteria play important roles in the shrimp paste fermentation process. In order to survive under the low water activity (A_w) conditions caused by the high salt concentration, the bacteria need to employ a special adaptation strategy. This study found that most halophilic bacteria isolated from shrimp paste accumulated ectoines (ectoine and hydroxyectoine) as protective osmotic agents. Five isolated bacteria, including three high ectoine producers and two high hydroxyectoine producers, were selected for further study. Based on their morphological and biochemical characteristics and 16S rRNA gene sequences, the five strains were classified into three genera: *Salinivibrio* (strains M7 and M316), *Salimicrobium* (strains M31 and M69), and *Vibrio* (strain M92). The accumulation of ectoines by *Salimicrobium* species is reported here for the first time. The effects of salinity, incubation temperature, and initial pH on the growth rate and accumulation of ectoines by the five strains were investigated. The results revealed that the bacterial growth rate was inhibited while the accumulation of ectoines by the five selected strains was triggered by an increase in the external salinity, incubation temperature, or initial pH. In addition, a high concentration of ectoine only (21.2 wt%) was produced by strain M316 at the optimum salinity and temperature, and under pressure of a high initial pH value. To the best of our knowledge, this is the first report demonstrating that the production of ectoines by bacterial strains can be enhanced by increasing the pH of the culture medium to induce pH stress. This finding suggests a new ectoine producer and fermentation strategy that may help to improve the production of ectoines in the future.

Introduction

Shrimp paste (*Mam tom*) is a traditional fermented product used as an ingredient in the cuisines of Vietnam and many other Asian countries [1, 2]. Shrimp paste is made from *Acetes* shrimp, mainly *Acetes japonicus* species. In order to produce shrimp paste, salt is mixed with ground shrimps at a concentration of 10–15% (w/w); the mixture is then transferred to jars and fermented for at least 4 weeks [3]. During the fermentation, the mixture is periodically stirred and exposed to sunlight. Within the temperature range of 30–40 °C, shrimp proteins are degraded into short chain peptides and free amino acids by enzymes and microorganisms from the shrimp. In addition, lipolysis reactions take place during the fermentation process. Hydrolytic products such as peptides, amino acids, and fatty acids are important contributors to the development of the typical taste as well as the flavor of shrimp paste, and they also exhibit strong antioxidant activities [4, 5].

Microorganisms are important factors that play dominant roles in the shrimp paste fermentation process. They contribute to the degradation of proteins, lipids, and other macromolecules, resulting in the development of the characteristic flavor and aroma of the shrimp paste product. The microorganisms found in shrimp paste may be derived from the shrimp or from contaminants introduced during handling or processing, and the halophilic bacteria count is known to increase during the fermentation process. There are also many proteolytic, lipolytic, and lactic acid bacteria present. Sodium chloride (NaCl) plays a key role in preservation by inhibiting the growth of spoilage and pathogenic bacteria, and inducing the growth of halophilic bacteria [6].

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Published online: 08 April 2021

During the fermentation process, the salting and drying steps lead to decreases in the water activity (A_w) of the shrimp paste. For example, after 30 days of fermentation, A_w of *Kapi* (a shrimp paste product of Thailand) decreased to 0.694 [6]. To survive in this low A_w environment, the bacteria need to develop osmotic adaptation strategies to reduce the intracellular water activity. Most bacteria accumulate compatible solutes that can balance the osmotic pressure differential between the cells and the surrounding medium [7]. Previous studies have shown that ectoines [ectoine and its hydroxyl derivative, hydroxyectoine (H-ectoine)] are the dominant compatible solutes accumulated by heterotrophic halophilic bacteria [8, 9].

Ectoines have been widely used in biotechnology as protective agents for enzymes, DNA, and whole cells against stress conditions [10–13]; ectoines are also used as a moisturizer in cosmetics or skin care products [14, 15]. And recently, ectoines have also been used in medical and health-care products such as eye drops and nasal sprays for the treatment of dry eye and dry nose and dermatological creams for the treatment of atopic dermatitis and other inflammatory skin diseases [16, 17].

This study investigated the accumulation of typical compatible solutes (ectoine and H-ectoine) by halophilic bacterial species isolated from shrimp paste (a high salinity environment that has not been well defined) to cope with the low A_w environment. The selected ectoine-producing strains were identified by molecular analysis of their 16S rDNA sequences and phenotypic characterization. Furthermore, the effects of different culture conditions such as salt concentration, initial pH, and incubation temperature on the growth rates and yields of ectoines in the selected strains were also evaluated. These data will explain how halophilic bacteria can survive under changing stress conditions during the fermentation process and suggest ways to control the culture conditions in order to get high yields of ectoines.

Materials and Methods

Isolation of Bacterial Strains

Shrimp paste (*Mam tom*) produced traditionally from the Hai Hau district, Nam Dinh Province, was collected and used for this study. The samples were serially diluted with sterile 10% NaCl solution, and then 100 µL of the diluted paste was spread on modified LB (MLB) medium containing (g/L): yeast extract, 5; peptone, 5; NaCl, 100; and granulated agar, 20; and the pH was adjusted to 7. The petri dishes were incubated at 35 °C for 2 days. The bacterial colonies were collected by plating them again on fresh agar medium.

Screening of Ectoine-producing Bacteria

Bacterial isolates were grown on the solid MLB medium containing different salt concentrations (3%, 6%, 9%, 12%, 15%, 18%, and 21%) at 35 °C for 2 days. The isolated strains that grew well at different salt concentrations were chosen for further studies. The selected strains were then grown on liquid MLB medium containing 10% NaCl with rotary shaking at 180 rpm. After 30 h of cultivation at 35 °C, samples were withdrawn for cell dry weight (CDW) determination and analysis of the ectoines contents.

Identification of Ectoine-producing Bacteria

Morphological characterization was performed using scanning electron microscopy (S-4800, Hitachi, Tokyo, Japan) [18]. Bacterial identification was also achieved by biochemical characterization. For phylogenetic studies, the 16S rRNA genes of the bacteria were amplified by PCR using universal primers: 314F (5′-CCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′), and 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Sequencing of the amplified DNA fragment was performed at 1st Base (Singapore). GenBank and Ribosomal Database Project databases were used to determine 16S rRNA gene similarities. Phylogenetic analysis based on the 16S rRNA gene was performed with the aid of the Mega six software package [19], using the neighbor-joining distance correction methods [20]. To construct the phylogenetic tree, only sequences from type strains of species whose names have been validated in publications were taken into account. Almost complete sequences (about 1400 bp) of the 16S rRNA genes of the strains isolated in Vietnam were deposited at the GenBank/EMBL/DDBJ databases (accession numbers for strains M7, M31, M69, M92, and M316 were MH938322, MH938323, MH938324, MH938325, and MH938326, respectively), and were used in the analysis.

Influences of Different Culture Conditions

The influences of salinity, incubation temperature, and initial pH on the growth rate and ectoine production by the five selected strains were investigated by using the "one variable at a time" method. The bacterial strains were first grown on liquid LB medium containing different salt concentrations (0%, 3%, 6%, 9%, 12%, 15%, 18%, and 21%) at 30 °C and pH 7. In the second experiment, the five strains were grown at the optimum salt concentration, pH 7, and at different incubation temperatures (25, 30, 35, and 40 °C). And in the third experiment, the strains were

grown at the optimum salt concentration and incubation temperature, but at different initial pH values (5, 6, 7, 8, 9, and 10). After 30 h of cultivation with rotary shaking at 180 rpm, samples were withdrawn for CDW determination and ectoine content analysis. All of the experiments were carried out at least in triplicate.

Quantitative Analysis

CDW was determined by centrifuging 3 mL of each culture sample at 6000 g for 10 min in a pre-weighed centrifuge tube, and the pellet was then washed once with 3 mL distilled water, centrifuged, and dried at 105 °C until constant weight was obtained. The centrifuge tube was weighed again to calculate the CDW.

Extraction of compatible solutes for ectoine analysis was performed as described previously [21]. A 10-mg cell mass was extracted with 570 µL of extraction mixture (methanol/chloroform/water 10:5:4, by volume) by vigorously shaking for 5 min followed by the addition of equal volumes (170 µL) of chloroform and water. The mixture was shaken again for 10 min and phase separation was enhanced by centrifugation. The hydrophilic top layer containing the ectoines was recovered. Concentrations of ectoines were determined by high-performance liquid chromatography [22], using an UltiMate 3000 Standard Dual System with an Aminex HPX-87C column (Biorad) and a UV detector at 65 ºC, with detection of the compounds at 210 nm. Calcium chloride (5 mM) was used as the mobile phase at a flow rate of 0.3 mL/min. Ectoine and H-ectoine (Sigma) were used as standards for calibration. Analysis was performed in triplicate for all samples.

The intracellular ectoines contents per gram biomass (weight percent, wt%) and the total ectoines concentrations per liter culture broth were determined.

The chemical structures of the compatible solutes accumulated in the selected bacterial strains were determined by proton nuclear magnetic resonance (^{1}H-NMR) analysis. For this, the bacterial strains were grown in shake flasks at 35 °C for 48 h. Cells were harvested by centrifugation (6000 g for 10 min) and lyophilized. The compatible solutes were extracted from the lyophilized cells as reported previously [21] and were also lyophilized. Fifteen milligrams of the freeze-dried sample were dissolved in 0.75 mL of D_2O for ^{1}H-NMR analysis on a Bruker DRX-600 NMR spectrometer (^{1}H 600 MHz).

Statistical analysis was performed using SPSS software version 20.0. Data were expressed as means ± standard deviation (SD). Comparisons between groups were performed using one-way ANOVA (LSD test). A value of $P < 0.05$ was considered statistically significant.

Results

Isolation of the Bacteria and Screening of Ectoine-Producing Strains

Shrimp paste samples were serially diluted and inoculated on MLB medium. After 2 days of incubation at 35 °C, several hundreds of bacterial colonies were obtained on agar plates. About 350 bacterial colonies were collected and grown on fresh agar medium. Of the bacterial strains grown on MLB medium containing different NaCl concentrations from 3 to 21%, 17 that grew at all tested salt concentrations were selected.

The abilities of the 17 selected bacterial strains to accumulate ectoines were tested and are shown in Table 1. Only the bacterial strain M204 was unable to accumulate ectoines; the remaining 16 bacterial strains were observed to accumulate ectoines with the contents ranging from 0.8 to 15.3 wt%. The peaks in the NMR spectra showed that ectoine and H-ectoine were the two dominant compatible solutes accumulated by the selected halophilic bacterial strains (Fig. 1a and b). Among these, three strains (M7, M92, and M316) that exhibited high ectoine contents above 10 wt% (Fig. 1a) and two other strains (M31 and M69) that accumulated both ectoine and H-ectoine (Fig. 1b) were selected for further studies. The five selected strains have been deposited at the Vietnam Type Culture Collection (VTCC) (accession numbers for strains M7, M31, M69, M92, and M316 were VTCC 910099, VTCC 910100, VTCC 910101, VTCC 910102, and VTCC 910103, respectively).

Identification of the Selected Bacterial Strains

The morphological and physiological characteristics of the five selected strains are summarized in Table 2. Three strains (M7, M92, and M316) were Gram negative, motile, and curved rod shaped (Supplementary Figure 1a, d, and e), whereas the other two strains (M31 and M69) were Gram positive, spherical shaped, and occurred either singly or in pairs (Supplementary Figure 1b and c). All five strains were non-spore-forming bacteria. They were mesophilic bacteria with optimum growth temperatures between 30 and 32 °C and grew well at neutral pH. The optimum salt concentrations for strains M7, M31, M69, and M316 were between 9 and 12%, and for strain M92, it was 3–6%. Moreover, all five strains gave positive catalase. These strains could utilize some simple carbon sources such as glucose and sucrose (Table 2).

The phylogenetic characteristics of the five selected bacterial strains were analyzed using their 16S rRNA

Table 1 Bacterial growth and ectoines accumulation by 17 selected strains

Strains	CDW (g/L)	Ectoine content (% CDW)	H-ectoine content (% CDW)	Total ectoines concentration (g/L)
M7	1.24 ± 0.04	14.7 ± 0.3	0	0.18
M20	1.74 ± 0.07	6.7 ± 0.2	0	0.12
M31	1.75 ± 0.11	1.1 ± 0.1	6.3 ± 0.2	0.13
M65	1.50 ± 0.09	6.0 ± 0.2	0	0.09
M69	1.00 ± 0.03	1.7 ± 0.1	5.5 ± 0.1	0.07
M83	1.49 ± 0.04	6.0 ± 0.3	0	0.09
M91	1.54 ± 0.05	6.3 ± 0.1	0	0.10
M92	2.60 ± 0.50	10.6 ± 0.2	0	0.28
M94	2.36 ± 0.05	7.5 ± 0.3	0	0.19
M95	1.60 ± 0.06	6.5 ± 0.1	0	0.12
M96	1.69 ± 0.07	5.4 ± 0.2	0	0.11
M101	2.39 ± 0.10	7.1 ± 0.4	0	0.13
M204	1.31 ± 0.08	0	0	0
M315	1.40 ± 0.07	9.3 ± 0.3	0	0.13
M316	1.24 ± 0.05	15.3 ± 0.2	0	0.19
M320	1.17 ± 0.03	1.7 ± 0.1	0	0.02
M327	2.50 ± 0.03	0.8 ± 0.1	0	0.02

gene sequences, and they were classified into three groups (Fig. 2). Strains M7 and M316 clustered together and exhibited a partial 16S rDNA similarity of 99.9%. The sequences of these two strains shared a close relationship with those of *Salinivibrio* spp., and the similarity with *Salinivibrio costicola* DSM 8285 was the closest (99%). The sequence of strain M92 displayed a high level of similarity (99.1%) with that of *Vibrio alginolyticus* ATCC 17749. The two remaining strains, M31 and M69, also clustered together and showed the highest similarity of 99% with *Salimicrobium jeotgali* KACC 16972 (Fig. 2).

Influences of the Different Culture Conditions on Growth Rates and Accumulation of Ectoines by the Five Selected Strains

Influence of Different NaCl Concentrations

The influence of different NaCl concentrations on the growth rates and accumulation of ectoines of the five selected strains are shown in Fig. 3. Strains M7, M92, and M316 grew and accumulated ectoines in the media containing 0–18% NaCl. At 12% NaCl, strains M7 and M316 gave the highest CDWs of 4.1 g/L and 3.5 g/L, respectively, whereas the maximum CDW of 3.9 g/L was obtained from strain M92 at 6% NaCl (Fig. 3a). The maximum ectoine contents of 17.2 wt%, 16.6 wt%, and 17.9 wt% were obtained at 12% NaCl from strains M7, M92, and M316, respectively (Fig. 3b). The highest H-ectoine contents of 0.3, 1.6, and 1.5 wt% were produced by strains M7 (12% NaCl), M92 (15% NaCl), and M316 (18% NaCl), respectively (Fig. 3c). The total ectoine contents, including ectoine and H-ectoine, accumulated by strains M7, M92, and M316 increased with increasing NaCl concentration in the initial culture medium and reached maximum values of 17.5 wt% (strain M7), 17 wt% (strain M92), and 17.9 wt% (strain M316) at 12% NaCl, but then declined at higher NaCl concentrations (Fig. 3d).

The other two strains, M31 and M69, were unable to grow in the absence of NaCl. They grew at a wide range of salt concentrations from 3 to 21% and, at a NaCl concentration of 9%, strains M31 and M69 showed maximum CDWs of 2.7 g/L and 2.5 g/L, respectively (Fig. 3a). The two strains accumulated low ectoine contents at NaCl concentrations of 9% or lower, but the yields increased when higher salt concentrations were provided and reached maximum values of 12.3 wt% (strain M31) and 14.2 wt% (strain M69) at 15% and 18% NaCl, respectively (Fig. 3b). In contrast, at 9% NaCl, the highest H-ectoine contents of 5.9 wt% and 7.4 wt% were achieved by strains M31 and M69, respectively (Fig. 3c). In addition, the total ectoine contents also increased with increasing NaCl concentration in the culture medium, and the maximum ectoine contents of 12.9 wt% and 14.7 wt% were obtained by strains M31 and M69 at 15% and 18% NaCl, respectively (Fig. 3d).

Influence of Different Incubation Temperatures

The five strains were grown on liquid MLB medium containing the optimum salt concentration for each strain and at different temperatures. Figure 4a shows that high CDWs

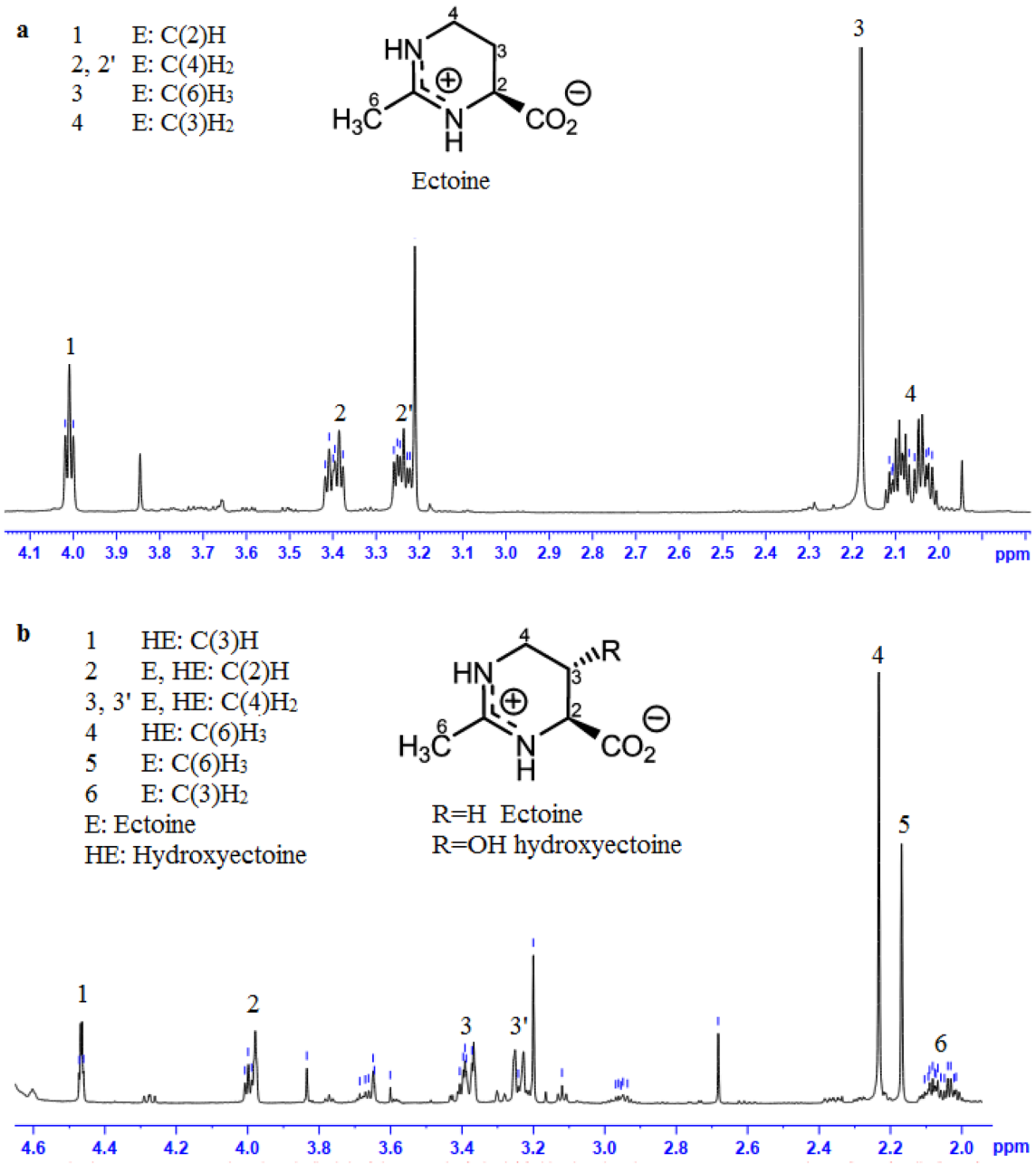

Fig. 1 ^{1}H-NMR spectra of compatible solutes extracts from strain M316 (**a**) and strain M69 (**b**) grown at 10% NaCl, with signals from ectoine (E) and H-ectoine (HE)

were achieved by the strains at temperatures between 25 and 30 °C. Maximum CDWs of 4.3, 2.7, 2.6, 4.1, and 3.7 g/L were achieved by strains M7, M31, M69, M92, and M316, respectively, at 30 °C. The cell masses then decreased when the temperature was increased to 35 and 40 °C. Changing the temperature from 25 to 35 °C did not affect the accumulation of ectoine by strains M31, M69, M92, and M316, but a significant effect on the amount of ectoine accumulated by strain M7 was observed. Increasing the incubation temperature to 40 °C led to a reduction of ectoine content in the bacterial cells of strains M7, M69, M92, and M318 but induced the accumulation of ectoine in strain M31. Maximum ectoine contents of 18.2 wt%, 4.6 wt%, 3.4 wt%, 8.4 wt%, and 17.1 wt% were obtained by strains M7 (35 °C), M31 (40 °C), M69 (35 °C), M92 (30 °C), and M316 (30 °C), respectively (Fig. 4b). H-ectoine was not identified in the cells of strain M92 at any tested temperature. Changing the temperature from 25 to 35 °C showed no significant effect on the accumulation of H-ectoine by strains M7 and M316, but the amount of H-ectoine increased to maximum values of 1.4

Table 2 Morphological and biochemical characteristics of the five selected strains

	M7	M31	M69	M92	M316
Morphological characteristic					
Shape	Curved rod	Cocci	Cocci	Curved rod	Curved rod
Size	0.5–0.8 × 0.7–3.5	0.8–1.0	0.7–0.9	0.6–0.8 × 1.3–1.8	0.6–0.8 × 1.2–1.5
Flagellum	+	−	−	+	+
Gram staining	−	+	+	−	−
Spore formation	−	−	−	−	−
Growth conditions					
Optimum temperature (°C)	30–32	28–30	28–30	30–32	28–30
Optimum pH	6–7	7–8	7–8	6–7	6–7
Optimum NaCl (%, w/v)	9–12	9–12	9–12	4–6	9–12
Biochemical characteristic					
Catalase	+	+	+	+	+
Urease	−	−	−	+	+
Lipase	−	−	−	−	+
D-mannitol	+	−	+	+	−
D-maltose	+	−	+	+	+
D-glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
L-arabinose	+	−	−	+	+
D-arabitol	+	−	−	+	+
D-trehalose	+	−	−	+	+
L-rhamnose	+	−	−	+	−
Inositol	+	−	−	−	−
D-cellobiose	+	−	−	+	+
D-sorbitol	+	−	−	+	+

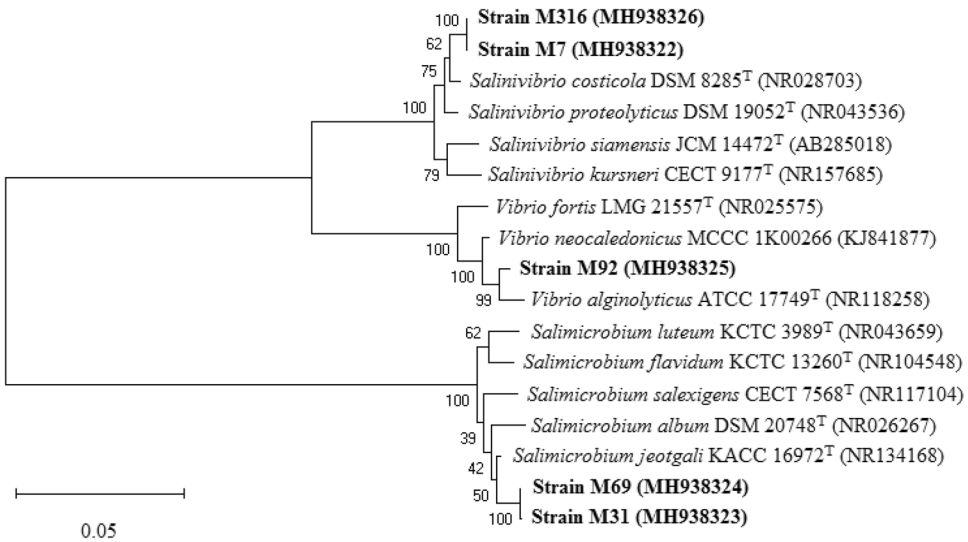

Fig. 2 Neighbor-joining phylogenetic tree based on the comparison of 16S rRNA gene sequences, showing the relationships between the five selected bacterial strains and other strains of the genera *Salinivibrio*, *Vibrio*, and *Salimicrobium*. Bar, five subtitutions per 1000 nucleotides

wt% (strain M7) and 1.3 wt% (strain M316) at 40 °C. The amount of H-ectoine that accumulated in strains M31 and M69 depended on the incubation temperature: maximum H-ectoine contents of 6.4 wt% and 7 wt% were obtained by strains M31 and M69, respectively (Fig. 4c). In summary, changing the incubation temperature led to changes in the amounts of accumulated ectoines in the cells of the five selected strains (Fig. 4d). The highest total ectoine contents of 10.3 wt%, 10.1 wt%, 8.4 wt%, and 17.1 wt% were obtained by the four strains M31, M69, M92, and M316, respectively, at the optimum growth temperature of 30 °C. Strain M7 was the only exception, as its optimum

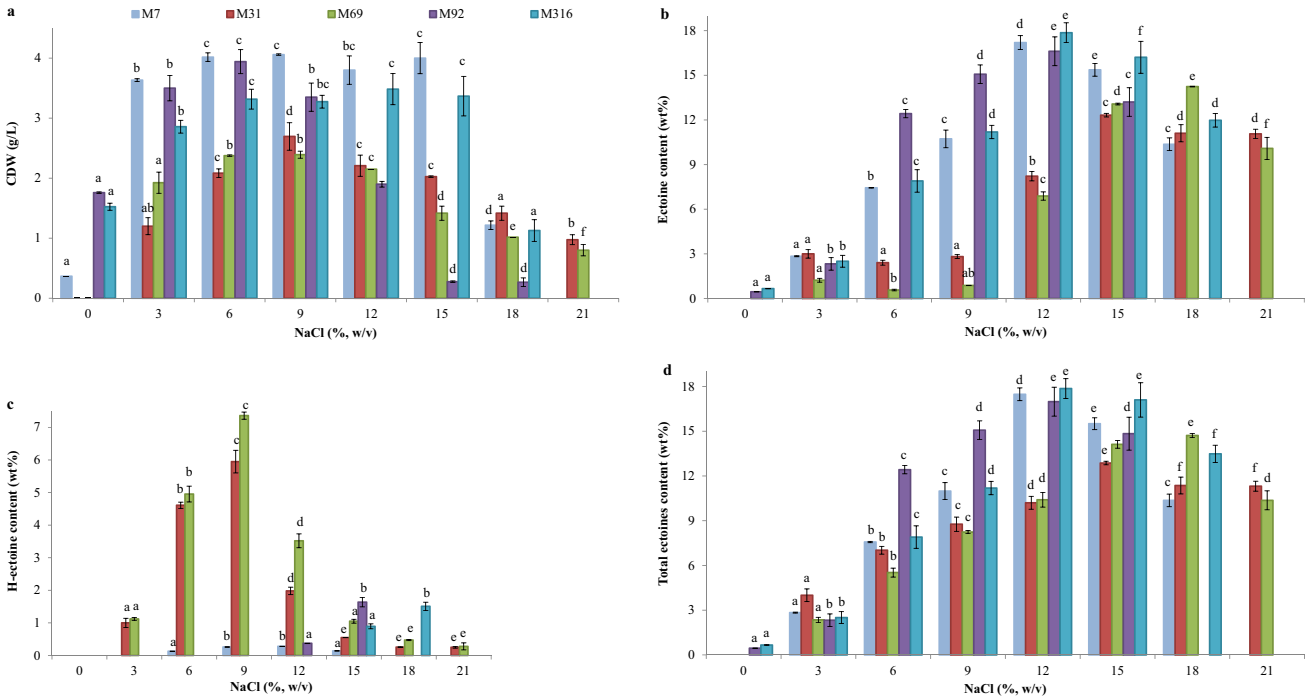

Fig. 3 Effects of NaCl concentration on cell dry weight (**a**), ectoine content (**b**), H-ectoine content (**c**), and total ectoines content (**d**) in the five selected bacterial strains grown in shake flasks at 30 °C and pH 7. Values are means and standard deviations ($n=3$). Bars with different letters within each bacterial strain are significantly different ($P<0.05$)

temperature for growth was 30 °C, but the highest ectoine content of 18.8 wt% was obtained at 35 °C (Fig. 4d).

Influence of Different Initial pH Values of the Medium

The five strains were then grown on liquid MLB medium containing the optimum salt concentration for each strain, at 30 °C and different initial pH values. Strains M31 and M69 could not grow at pH 5, but they grew well at pH 6–8 (Fig. 5a), and high ectoine contents of 7.5 wt% (strain M31) and 6.7 wt% (strain M69) were obtained at pH 10 (Fig. 5b), whereas the maximum H-ectoine contents of 5.2 wt% (strain M31) and 7.0 wt% (strain M69) were achieved at pH 7 (Fig. 5c). In addition, the maximum total ectoine contents accumulated by the two strains, M31 and M69, were obtained at pH 7: 9.9 wt% and 10.2 wt%, respectively (Fig. 5d).

Changing the initial pH of the culture medium from 5 to 10 produced negligible effects on the CDW and the accumulations of ectoine and H-ectoine by strain M92 (Fig. 5a, b, c, d) but did have significant effects on the growth rate and ectoine accumulation of strains M7 and M316 (Fig. 5a and b). Maximum CDWs of 5.4 g/L and 3.5 g/L were reached at pH 6 by strains M7 and M316, respectively (Fig. 5a). In contrast to the CDWs, the highest ectoine contents of 18.8 wt% and 21.2 wt% were obtained at pH 10 by strains M7 and M316, respectively (Fig. 5b).

Discussion

In saline environments, one of the key factors affecting microbial growth is the availability of free water. Recognizing and adapting to changes in the environmental conditions are critical processes that determine the survival of microorganisms in a given habitat [7]. The accumulation of compatible solutes is a typical strategy used by most of the halophilic bacteria to balance their cytoplasm osmotically against a highly saline environment [7, 8, 15]. Ectoines, including ectoine and H-ectoine, are among the most abundant osmolytes in nature [23]. The results obtained in this study agree with previous studies, as can be seen from Table 1, in which most of the selected bacterial strains isolated from fermented shrimp paste (16/17) displayed their potential to accumulate ectoines. In the medium containing 10% NaCl, 14-isolated strains accumulated ectoine and two strains, M31 and M69, accumulated both ectoine and H-ectoine (Table 1). To date, there have been some studies on microorganisms isolated from shrimp paste collected in the Asian region, e.g., Myanmar [24], Indonesia [5], and Thailand [25], and recently a new type strain *Lentibacillus lipolyticus* JCM 32625 harboring ectoine synthesis genes was isolated from Thailand [26]. In a previous report, a halophilic bacterium strain *Salinivibrio proteolyticus* M318 harboring genes for the synthesis of both ectoines and biopolymer polyhydroxyalkanoates was isolated from Vietnam [27]. However, this is

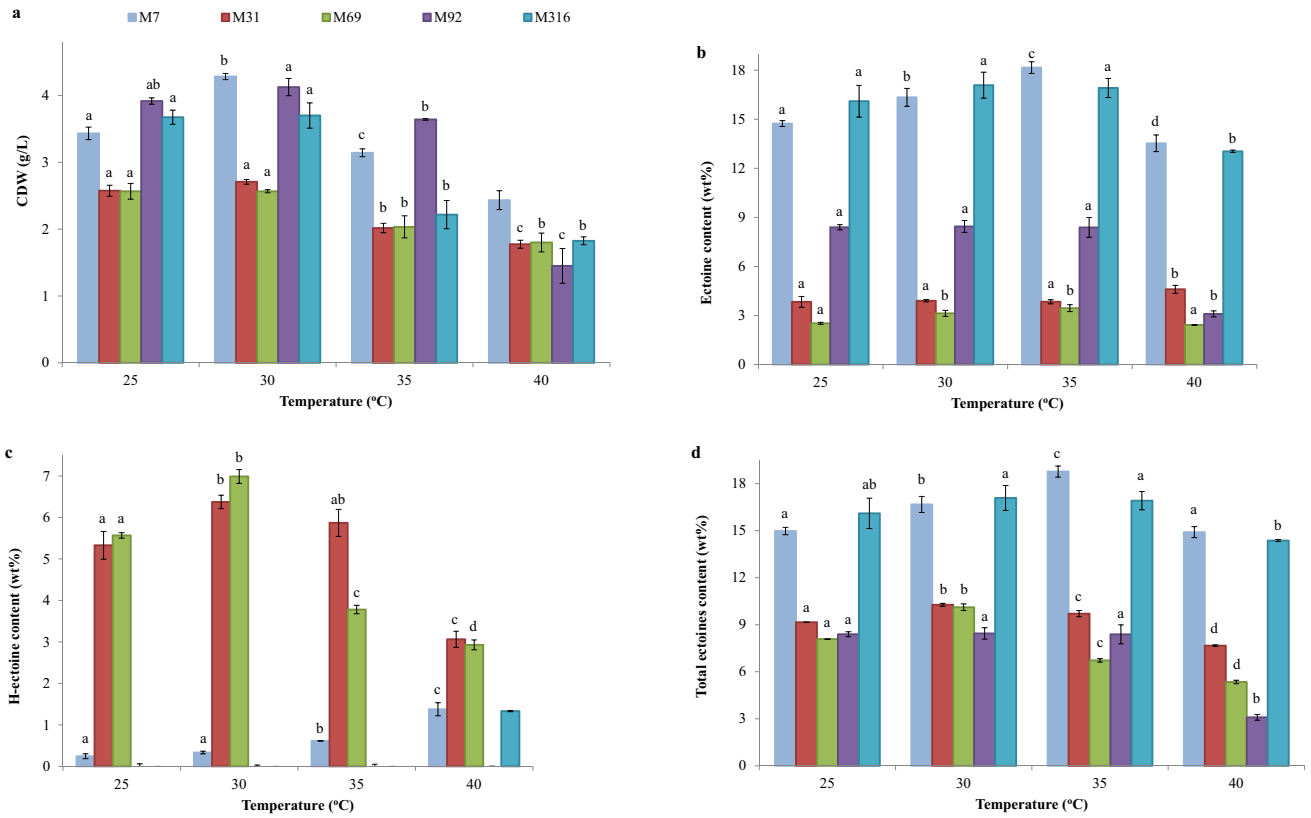

Fig. 4 Effects of incubation temperature on the cell dry weight (**a**), ectoine content (**b**), H-ectoine content (**c**), and total ectoines content (**d**) in the five selected bacterial strains grown in shake flasks at pH 7 and the optimum NaCl concentration for each strain. Values are means and standard deviations ($n=3$). Bars with different letters within each bacterial strain are significantly different ($P<0.05$)

the first study to report that ectoine and H-ectoine were the two dominant compatible solutes accumulated by most of the halophilic bacteria isolated from shrimp paste.

Analysis of their morphological and biochemical features and comparison of their 16S rRNA gene sequences with other published reference strains showed that the five selected strains can be identified with one of three genera: *Salinivibrio* (strains M7 and M316), *Vibrio* (strain M92), and *Salimicrobium* (strains M31 and M69) (Table 2 and Fig. 2). The genus *Salinivibrio* belongs to the family *Vibrionaceae* and currently includes only six species. They are all halophilic bacteria commonly found in salted foods, saline soils, and hypersaline aquatic environments. The strains of *Salinivibrio* are Gram-negative, non-spore-forming, curved rods, motile by one polar flagellum, and facultatively anaerobic [28, 29]. Compatible solutes such as glycine betaine, glutamate, and ectoine have been found in the cells of *Salinivibrio costicola* subsp. *yaniae*, and among them, ectoine performed a key role in osmotic adaptation to high salinity environments [30]. A maximum ectoine concentration of 0.455 g/L was obtained in strain *S. costicola* subsp. *yaniae* [30], whereas, the highest ectoine concentrations of 0.963 g/L and 0.6 g/L were obtained by strains *Salinivibrio* sp. M7 and *Salinivibrio* sp. M316, respectively [data not shown]. Recently, the complete cluster of genes responsible for ectoine synthesis (*ectABC*) from aspartate semialdehyde has been found in the genome sequences of *Salinivibrio* species: *S. costicola, S. kushneri*, *S. proteolyticus S. sharmensis*, and *S. siamensis* [27, 28]. This suggests that ectoine is the key compatible solute responsible for osmotic adaptation of salinivibrios. In addition, the gene for H-ectoine synthesis (*ectD*) from ectoine has also been found in the genome sequences of *Salinivibrio* species [27, 28].

Vibrio is a big genus of the family *Vibrionaceae*, with more than 100 species reported so far (https://lpsn.dsmz.de/genus/vibrio). Vibrios are Gram negative, curved rod shaped, motile by a single polar flagellum, and commonly found in aquatic environments. The genus can be divided into two groups: halo-tolerant and halophilic vibrios. Some of them cause disease in humans, such as *V. cholerae, V. parahaemolyticus*, and *V. vulnificus*, as well as in marine animals, but many of them are only occasionally implicated as opportunist pathogens [31]. Ectoine was reported as being synthesized by many species belonging to the genus *Vibrio* for osmotic adaptation [32–35] or cold stress adaptation [36], and the *ectABC* genes were also found in many *Vibrio*

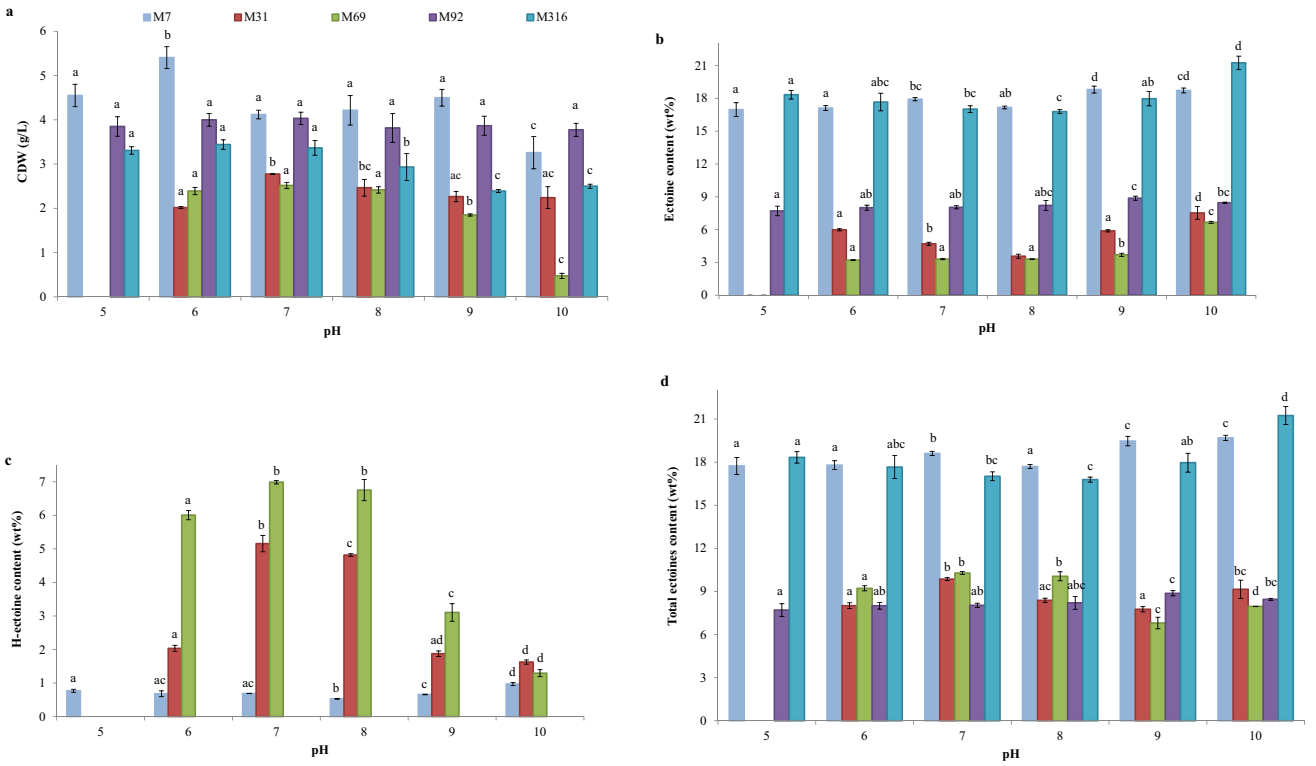

Fig. 5 Effects of the initial pH on the cell dry weight (**a**), ectoine content (**b**), H-ectoine content (**c**), and total ectoines content (**d**) in the five selected bacterial strains grown in shake flasks at 30 °C and the optimum NaCl concentration for each strain. Values are means and standard deviations ($n=3$). Bars with different letters within each bacterial strain are significantly different ($P<0.05$)

species: *V. parahaemolyticus, V. alginolyticus, V. harveyi, V. vulnificus, V. cholera, V. fischeri*, and *V. anguillarum* [34–36]. *Vibrio* species tend to accumulate mixtures of compatible solutes such as betaine, ectoine, and glutamate for osmotic adaptation [32, 35], and ectoine synthesis is critical for growth under osmotic stress conditions [35]. Based on the culture conditions, the ectoine content ranged from 0.5% to 7% of CDW [32].

The genus *Salimicrobium* includes six species: *S. album*, *S. halophilum*, *S. luteum* [37], *S. flavidum* [38], *S. salexigens* [39], and *S. jeotgali* [40]. The species of the genus *Salimicrobium* were isolated from saltern environments [38, 39] or fermented seafood [40]. They are Gram-positive, strictly aerobic, rod or cocci, moderately halophilic bacteria that require NaCl to grow, and exhibit optimum growth at around 10% NaCl [38–40]. At the time of writing, there has been no report on the osmotic adaptation of the species belonging to the genus *Salimicrobium.*

Abiotic factors such as salinity, temperature, and pH play major roles in determining the growth of bacteria in traditional fermentation products. These factors change during the fermentation process. Bacteria need to have mechanisms that allow them to recognize such changes and rapidly regulate their physiology and metabolism to cope with them [7]. Besides, their role as osmoprotectants, ectoine and H-ectoine also has potential to act as protective agents for DNA, enzymes, and entire cells against heat, cold, or pH stress [10, 11, 41]. Therefore, changing the culture conditions, such as NaCl concentration, temperature, and pH, may affect both the growth rates and intracellular ectoine concentrations in the bacterial cells. The results obtained in Figs. 3, 4, and 5 showed that the growth rates and the ectoine contents of the five selected strains were strictly dependent on the NaCl concentration, temperature, and initial pH, but the levels of influence were different among the tested factors and bacterial strains. The five selected strains grew well at a wide range of NaCl concentrations: 0–12% (strain 97) and 3–15% (strains M7, M31, M69, and M316) (Fig. 3a). The growth rates and total ectoine contents increased when the NaCl concentration in the culture medium increased, but if the NaCl concentration in the culture medium was too high, e.g., 18% and 21%, this led to decreases in both CDWs and total ectoine contents (Fig. 3a, d). Previous studies reported that high ectoine contents were normally synthesized when the ectoine-producing bacteria were grown at low or optimum salt concentration, and high H-ectoine contents were then synthesized when a high salt concentration was added to the culture medium [42, 43]. These trends were also observed in this study with the three strains, M7, M92, and M316, but not with the other two strains, M31 and M69. The highest

H-ectoine contents of 5.9 wt% and 7.4 wt% were synthesized by the two strains M31 and M69, respectively, when they were grown at the optimum salt concentration of 9%, while ectoine contents of only 2.8 wt% and 0.9 wt% were accumulated by strains M31 and M69, respectively. The H-ectoine contents and CDWs then decreased and ectoine contents increased when the salt concentration in the culture medium was increased (Fig. 3a, b, c). Similar results were obtained when these two strains were grown at different temperatures or initial pH values: the highest H-ectoine contents were achieved at the optimum growth temperature of 30 °C or the optimum growth pH of 7 (Figs. 4c and 5c), while increasing or decreasing the temperature or pH value resulted in decreasing both the CDW and H-ectoine contents (Figs. 4a, c, and 5a, c), but increasing the ectoine contents (Figs. 4b and 5b). This is a new finding suggesting that H-ectoine is the main compatible solute synthesized by the two strains M31 and M69 (that belong to the genus *Salimicrobium*) under optimum growth conditions, whereas a greater amount of ectoine is synthesized when the culture conditions become more stressful.

Changing the temperature from 25 to 35 °C and the initial pH from 5 to 10 had insignificant effects on the growth rate and ectoine accumulation by strain M92 (Figs. 4 and 5); only at 40 °C was a negative effect on both the CDW and ectoine content of strain M92 observed (Fig. 4a and b). The other two strains, M7 and M316, grew optimally at 30 °C, but the highest ectoine contents were obtained at 35 °C, while increasing the temperature to 40 °C resulted in decreases in both CDWs and ectoine contents (Fig. 4). The optimum growth pH for strains M7 and M316 was 6, but the highest total ectoine content of 19.8 wt%, including 18.8 wt% ectoine and 1 wt% H-ectoine (strain M7), and the highest ectoine content only, of 21.2 wt% (strain M316), were obtained at pH 10 (Fig. 5). These results suggest that the amounts of intracellular ectoines in the five selected halophilic bacteria are triggered by an increase in NaCl concentration, temperature, or initial pH value. There have been several studies on the regulation of ectoine and H-ectoine accumulation according to saline [42–44], temperature [45–47], and pH [48] conditions, but this is the first report demonstrating that the accumulation of ectoines is regulated by pH stress in the culture medium. These findings provide a novel strategy for fermentation technology that would improve the production of ectoines by halophilic bacteria.

In addition, the highest ectoine content of 21.2 wt% obtained in this study by strain M316 is comparable to that of the most efficient ectoine-producing bacteria reported so far such as *Halomonas elongate*¸ which accumulated a total ectoine content of 21.2 wt% including 20.8 wt% ectoine and 0.4 wt% H-ectoine at 15% NaCl [42], and *Halomonas boliviensis*, which accumulated 27.8 wt% ectoines including 14.8 wt% ectoine and 13 wt% H-ectoine at 18% NaCl [43]. The two *Halomonas* strains accumulated both ectoine and H-ectoine, whereas strain M316 produced only ectoine when cultivated at a lower NaCl concentration of 12% NaCl and at pH 10; hence, its product will be easier to purify. This demonstrates M316 is a promising alternative bacterium for the production of ectoine.

Conclusion

Halophilic bacteria belonging to three genera (*Salinivibrio*, *Vibrio*, and *Salimicrobium*) were isolated and identified from fermented shrimp paste collected in Vietnam. Most of the isolated halophilic bacteria accumulated ectoine and H-ectoine as compatible solutes for osmotic adaptation. The amounts of accumulated ectoines in selected bacterial strains were triggered by an increase in the external salinity, incubation temperature, or initial pH. High ectoine producers can be found in fermented shrimp paste.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-021-02481-1.

Acknowledgements The authors acknowledge the Hanoi National University of Education, Vietnam for providing infrastructure facilities.

Author Contribution DVT and TTL designed the research. DVT, TTL, and NTT performed the experiments and analyzed the data. DVT wrote the manuscript with input from TTL.

Funding This research did not receive any specific grant from funding agencies in the public, commercial, or nonprofit sectors.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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