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ORIGINAL ARTICLE

Identification and characterization of ectoine-producing bacteria isolated from Can Gio mangrove soil in Vietnam

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Abstract

Purpose The aim of this study was to characterize high ectoine-producing bacteria obtained from Can Gio mangrove soil samples in Vietnam.

Methods Ectoine-producing bacteria were isolated from mangrove soil samples. The selected strains were identified using 16S rDNA sequence analysis, and their biochemical characteristics were also examined. The ability to produce ectoine at different NaCl concentrations and the effect of osmotic downshock solution on ectoine's release rates and survival rates for the selected bacterial strains were investigated.

Results Among more than 200 bacterial colonies isolated from soil samples, two strains exhibiting highest ectoine production (strains D227 and D228) were chosen for further studies. Both strains D227 and D228 were identified as *Halomonas* spp. and were closely related to *Halomonas organivorans*, sharing 99.4% 16S rDNA sequence similarity. At 6% (*w*/*v*) NaCl concentration, strains D227 and D228 presented the highest cell dry weight (CDW) of 3.85 and 3.55 g/l, respectively. At 18% NaCl concentration, maximum total ectoine (ectoine and hydroxyectoine) production of 16.4 and 18.1 wt% was achieved by strains D227 and D228, respectively. After 30 min of incubation in downshock solution containing 5% NaCl, high bacterial survival rates of 96% and 98%, and ectoines release rates of 61% and 76% were obtained by strains D227 and D228, respectively.

Conclusions The accumulation and secretion of ectoine appear to be a typical adaptation strategy of some bacteria to survive under the changing saline conditions of mangrove ecosystem. To the best of our knowledge, this is the first report on ectoine production by halophilic bacteria isolated from mangrove soil. High ectoine-producing bacteria can be found in mangrove forest.

Keywords Can Gio mangrove · Compatible solutes · Ectoine · *Halomonas* · Halophilic bacteria

Introduction

Many microorganisms synthesize and/or uptake compatible solutes for cell survival under stress conditions. Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is one of the most abundant compatible solutes, intracellularly accumulated by many bacteria for protection from stress (Roberts 2005; Lentzen and Schwarz 2006), and has been receiving significant attention because of its potential biotechnological applications (Pastor et al. 2010). It can be used as a protective agent for enzymes against stress conditions such as heat, cold, and high or low pH (Lippert and Galinski 1992; Van-Thuoc et al. 2013). It has been reported that addition of ectoine retained more than 80% of lactic dehydrogenase activity and 100% of phosphofructokinase activity after 4 cycles of freezing and thawing (Lippert and Galinski 1992). In addition, ectoine has also been shown to protect xylanase against pH stress; the enzyme completely lost its activity after 10 h of incubation at pH 4.5, whereas, in the presence of ectoine, about 10% of the original activity was maintained at pH 4.5 and significantly higher activity was noted at high pH of 11 and 12 (Van-Thuoc et al. 2013). Furthermore, ectoine can also be used for DNA protection. In a previous study, Schröter et al. (2017) reported that ectoine can prevent DNA strand breaks caused by ionizing electron radiation.

Besides its role in protecting macromolecules, many studies have also shown that ectoine can be used for whole cell

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protection. It has been demonstrated that the growth inhibition induced by osmotic stress in *Escherichia coli* can be reversed by the addition of ectoine into the culture medium (Malin and Lapidot 1996). Ectoine has also been found to protect human skin cells from the effects of harmful UV radiation (Bünger and Driller 2004). Kanapathipillai et al. (2004) showed that ectoine strongly inhibits Aβ42 amyloid formation in vitro and reduces toxicity to human neuroblastoma cells, suggesting its potential role in treating Alzheimer's disease. Moreover, a recent clinical trial confirmed that ectoine-containing products reduced nasal and ocular symptoms in allergic rhinitis patients (Werkhäuser et al. 2014). Most of the high ectoine producers belong to the genus *Halomonas*. For instance, *Halomonas elongata* is currently used for large-scale production of ectoine (Kunte et al. 2014), *Halomonas salina* can secrete high quantities of ectoine into the culture medium at low salt concentration (Zhang et al. 2009), and *Halomonas boliviensis* can accumulate nearly 28% ectoines (ectoine and hydroxyectoine) in the cells during two-step fed-batch fermentation (Van-Thuoc et al. 2010).

Can Gio is one of the 24 districts of Ho Chi Minh City, Vietnam. Its total area is about 72,000 ha, with a land cover of 40,000 ha, and mangrove cover of 32,000 ha. The Can Gio mangrove forest covers the delta of Saigon, Dong Nai, and Vam Co Rivers. In January 2000, Can Gio was designated as the first Mangrove Biosphere Reserve in Vietnam under the Man and Biosphere Programme of the United Nations Education, Scientific, and Cultural Organization. In general, the soil water salinity in mangrove forests is dynamic and depends on the fresh water input. In the Can Gio mangrove forest, water salinity is high during dry season, reaching peaks of 2.5–3% in March and April, but decreases during the rainy season to only 0.5–1% (Tuan and Kuenzer 2012; Costa-Böddeker et al. 2017). Many different microorganisms, including bacteria, fungi, protozoa, and algae, have been found in mangrove ecosystems (Holguin et al. 2001; Van-Thuoc et al. 2012). To survive under dynamic water salinity, microorganisms may accumulate compatible solutes such as ectoine for maintaining osmotic balance. In the present study, production of ectoine by bacterial species isolated from Can Gio mangrove soil was investigated.

Materials and methods

Samples collection and isolation of bacterial strains

Soil samples were collected from the surface of the Can Gio mangrove forest in Southern Vietnam. The samples were immediately placed in a sealed container and transported to the laboratory. A total of 1 g of soil sample was inoculated into meat peptone agar (MPA) medium containing (g/l) peptone, 5; meat extract, 5; and NaCl, 150. After 5 days of cultivation on a rotary shaker at 30 °C and 180 rpm, 1 ml of the culture broth was serially diluted with 15% NaCl solution, and 100 μl of the diluted culture broth were spread onto MPA plates. The plates were incubated at 30 °C for 5 days. More than 200 colonies were isolated and re-inoculated onto fresh MPA plates to obtain pure cultures.

Screening of ectoine-producing bacterial strains

Bacterial isolates were grown on MPA plates containing varying NaCl concentrations ranging from 3 to 21% at an increment of 3% (*w*/*v*). After 48 h of incubation, the bacterial strains that grew well in a wide range of salt concentrations were selected. The selected bacterial strains were then grown in MPA medium containing 15% NaCl on a rotary shaker at 30 °C and 180 rpm. After 30 h of cultivation, samples were collected for cell dry weight (CDW) and ectoine analysis.

Phylogenetic characterization of the selected ectoine-producing bacteria

The genomic DNA of the selected strains was extracted by a Thermo Scientific GeneJET Genomic DNA Purification Kit according to the manufacturer's recommendations. The 16S rDNA gene was amplified using the universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGCTT-3′). Amplification was performed with the following PCR conditions, initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1.5 min, followed by a final extension at 72 °C for 7 min. Sequencing of the amplified DNA fragment was performed at 1st Base (Singapore), and GenBank database was used to search for 16S rDNA similarities. Phylogenic analysis based on 16S rDNA was performed with the aid of MEGA6 software (Tamura et al. 2013) using the neighbor-joining distance correlation method. The almost complete sequences (about 1400 bp) of the 16S rDNA genes of the strains isolated from the Can Gio mangrove forest were deposited in GenBank/EMBL/DDBJ databases (Accession numbers for strain D227 is MH715408 and strain D228 is MH715409) and used in the analysis.

Morphological characteristics

The shape and size of the selected bacterial strains were determined by scanning electron microscopy (SEM). After 24-h cultivation in liquid medium, the bacterial cells were collected by centrifugation, washed thrice with 0.1-M phosphate-buffered saline, and fixed with 2.5% glutaraldehyde for 6 h and 1% osmic acid solution for 6 h. The samples were dehydrated in 50%, 70%, 85%, 95%, and 100% ethanol solution. Subsequently, ethanol was replaced with isoamyl acetate, and the samples were dried with carbon dioxide. Lastly, the

samples were sputter-coated with gold for 2 min and examined by SEM (S-4800, Hitachi, Japan).

Biochemical tests

The biochemical properties of the selected bacterial strains were determined using an ID 32 E kit (BioMérieux), which is a standardized system for the identification of *Enterobacteriaceae* and other non-fastidious Gram-negative rods. The kit comprises 32 miniaturized biochemical tests and a specific database.

Effects of salt concentration on growth and ectoine accumulation of the selected strains

The selected strains were grown in modified liquid HM (Medium for Halophile) containing (g/l): $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.009; KCl, 0.05; K_2HPO_4, 0.5; $FeSO_4 \cdot 7H_2O$, 0.005; monosodium glutamate, 2; glucose, 10; and different NaCl concentrations, 0%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%,12%, 15%, and 18%. After 30 h of cultivation at 30 °C and 180 rpm, the bacterial cells were collected by centrifugation for CDW and ectoine analysis.

Ectoine production in two-step fermentation

The selected bacteria were first grown in 75 ml of MPA medium containing 6% NaCl in 250-ml Erlenmeyer flasks on a rotary shaker at 30 °C and 180 rpm for 15 h. The bacterial cells were harvested from the culture broth by centrifugation at 5000×*g* for 10 min and then suspended in 75 ml of HM with varying concentrations of NaCl (18%, 21%, 24%, and 27%) in 250-ml Erlenmeyer flasks for ectoine production. After 24 h of cultivation, the bacterial cells were collected by centrifugation for CDW and ectoine analysis.

Determination of ectoine release and bacterial cell survival after osmotic downshock

The selected bacterial strains were grown in MPA medium containing 6% NaCl at 30 °C and 180 rpm for 15 h and then collected from the culture broth by centrifugation at 5000×*g* for 10 min. The pellet was suspended in fresh MPA medium containing 18% NaCl. After 24 h of cultivation, the cells were harvested by centrifugation and suspended in either distilled water or solutions containing 2.5% and 5% NaCl for ectoine release. After 30 min, the suspensions were centrifuged at 10,000×*g* for 10 min. The supernatants containing released ectoine were analyzed by high-performance liquid chromatography (HPLC). The cell pellet obtained after centrifugation was suspended in 6% NaCl solution, serially diluted with the same solution, and spread onto MPA plates containing 6% NaCl. The bacterial cells obtained from the culture broth without being subjected to osmotic downshock were also serially diluted and spread onto MPA plates. The bacterial colonies on the plates were counted after 48 h of cultivation at 30 °C. The percentage of cell survival was calculated based on the ratio of the number of colonies found after osmotic downshock and the number of colonies formed without osmotic downshock.

Determination of CDW

To determine the CDW of the selected bacterial strains, 3 ml of the culture samples were centrifuged at 5000×*g* for 15 min in a pre-weighed centrifuge tube, and the pellet was quickly washed with 3 ml of distilled water, centrifuged, and dried at 105 °C until a constant weight was obtained. The centrifuge tube was weighed again to calculate the CDW.

Ectoine analysis

Extraction of ectoine was performed as reported previously (Kunte et al. 1993). In brief, 10 mg of cell mass were extracted with 570 μl of extraction mixture (methanol/chloroform/water, 10:5:4 *v*/*v*) by vigorous shaking for 5 min, followed by 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 compatible solutes was recovered, and the concentration of ectoine was determined by HPLC (Onraedt et al. 2005) using an UltiMate 3000 Standard Dual System with an Aminex HPX-87C column (Bio-Rad) and a UV detector at 65 °C. The compounds were monitored at 210 nm, and calcium chloride (5 mM) was used as a mobile phase at a flow rate of 0.3 ml/min, with ectoine and hydroxyectoine (Sigma) employed as a standard for calibration. The intracellular ectoine content (g ectoine per g biomass, percentage by weight—wt%) and total ectoine concentration (ectoine per liter culture broth, g/l) were calculated according to standard procedures (Van-Thuoc et al. 2010).

Results

Screening of ectoine-producing bacteria

The pure cultures isolated from mangrove soil samples were grown on MPA plates containing different NaCl concentrations (from 3 to 21%) at 30 °C. After 24-h incubation, 12 bacterial strains that grew well in a wide range of salt concentrations were selected for further experiments. The selected strains were named D10, D16, D25, D30, D90, D91, D98, D209, D227, D228, D242, and D243. To test the ability of the selected strains to produce ectoine, the strains were grown in MPA medium containing 15% NaCl at 30 °C for 30 h.

Then, the cells were harvested for CDW and ectoine analysis. As shown in Table 1, the 12 selected strains grew well in MPA medium. The highest CDW of 1.82 g/l was exhibited by strain D98, whereas the lowest CDW of only 1.11 g/l was presented by strain D90. Interestingly, all the selected strains could produce ectoine ranging from 2.2 to 9.9 wt%. Among them, two strains, D227 and D228, accumulated the highest ectoine contents of 9.1 and 9.9 wt% respectively, and were selected for further analyses.

The phylogenetic characteristics of the two selected bacterial strains were analyzed using their 16S rDNA sequences. Strains D227 and D228 clustered together and presented a 16S rDNA sequence similarity of 100%. The sequences of these strains shared a close relationship with those of *Halomonas* spp. and showed closest similarity with *Halomonas organivorans* (99.4%), *Halomonas koreensis* (98.9%), and *Halomonas beimenensis* (97.9%) (Fig. 1).

Phenotypic characterization of the selected strains

The morphological and biochemical characteristics of the two selected strains are summarized in Table 2.The two selected strains were aerobic, Gram-negative, non-spore-forming, rod-shaped, and occurred either singly or in pair (Fig. 2A, B). They were both moderately halophilic bacteria with optimum salt concentration of 5–6% (*w*/*v*). Besides, the strains were also mesophilic with optimum temperatures for growth between 30 and 35 °C and grew well at pH between 6 and 7 (Table 2). Moreover, both the strains were positive for catalase and indole tests, but were lipase- and urease-negative. The strains could utilize many of the tested compounds, including potassium 5-ketogluconate, 4-nitrophenyl-βD-glucopyranoside, D-mannitol, D-maltose, adonitol, palatinose, 4-nitrophenyl-βD-galactopyranoside, D-glucose, sucrose, L-arabinose, D-arabitol, 4-nitrophenyl-αD-glucopyranoside, 4-nitrophenyl-αD-galactopyranoside, D-trehalose, L-rhamnose, inositol, D-cellobiose, D-sorbitol, and 4-nitrophenyl-αD-maltopyranoside. However, both the strains could not use L-ornithine, L-arginine, L-lysine, L-arabitol, sodium pyruvate, 4-nitrophenyl-βD-glucuronide, 5-bromo-4-chloro-3-indolyl-N-acetyl-βD-glucosaminide, and L-aspartic acid 4-nitroanilide. Although many of the characteristics of the two selected strains were obviously similar to those of *H. organivorans* CECT 5995^T, some features, such as the ability to use compounds, e.g. galacturonic acid, D-maltose, adonitol, L-tryptophan, and L-arabinose, were different between the two strains and *H. organivorans* (Table 2).

Table 1 Bacterial growth and ectoine accumulation by 12 selected strains

Strain	CDW (g/l)	Ectoine content (wt%)	Ectoine conc. (g/l)
D10	1.77	6.0	0.11
D16	1.55	6.2	0.1
D25	1.48	4.4	0.07
D30	1.78	5.9	0.11
D90	1.11	4.3	0.05
D91	1.49	5.4	0.08
D98	1.82	2.2	0.04
D209	1.6	7.4	0.12
D227	1.24	9.1	0.11
D228	1.26	9.9	0.12
D242	1.26	6.0	0.08
D243	1.43	5.5	0.08

Ectoine production by the selected strains at different NaCl concentrations

Investigation of the effect of different NaCl concentrations on the growth and ectoine accumulation of the two strains isolated from the Can Gio mangrove forest showed that the strains were unable to grow in the absence of NaCl. Salt concentrations between 4 and 7% were optimal for the growth of the two isolates, and their growth rapidly decreased at NaCl concentrations higher than 9%. Furthermore, at 6% NaCl concentration, strains D227 and D228 presented maximum CDW of 3.85 and 3.55 g/l, respectively (Fig. 3). However, the optimal salt concentration for cell growth was not favorable for ectoine accumulation. Ectoine accumulation increased with the increasing NaCl concentration in the initial culture medium. At 15% NaCl concentration, strains D227 and D228 exhibited highest ectoine production, reaching 11.26 and 14.07 wt%, respectively (Fig. 3). Besides, although the two strains were found to grow at a higher NaCl concentration of 18%, the obtained CDW was not sufficient for ectoine analysis.

Two-step fermentation was performed to investigate the effect of high concentration of NaCl on ectoine production by the two selected strains. After 15 h of growth in MPA medium containing 6% NaCl, the CDW of strains D227 and D228 reached 1.2 and 1.0 g/l with ectoine contents of 4.6 and 4.9 wt%, respectively (Fig. 4a–c). Subsequent incubation of the strains in HM containing 18%, 21%, 24%, and 27% NaCl revealed that the two strains could grow in the presence of 18% and 21% NaCl, but were destroyed at higher NaCl concentrations of 24% and 27%. Maximum CDW of 2.3 g/l was exhibited by strain D227 in the presence of 18% NaCl, which reduced to only 0.8 g/l at 27% NaCl concentration. Similar results were also noted for strain D228, with maximum CDW of 2.2 g/l obtained at 18% NaCl concentration, and only 0.5 g/l CDW observed in the presence of 27% NaCl (Fig. 4a). Maximum ectoine content of 16.5 wt% (14.5 wt% ectoine and 2 wt% hydroxyectoine) was achieved by strain D227 in the presence of 18% NaCl, and the hydroxyectoine content slightly increased from 2 to 2.4 wt% when the NaCl concentration increased from 18 to 21%. However, the ectoine

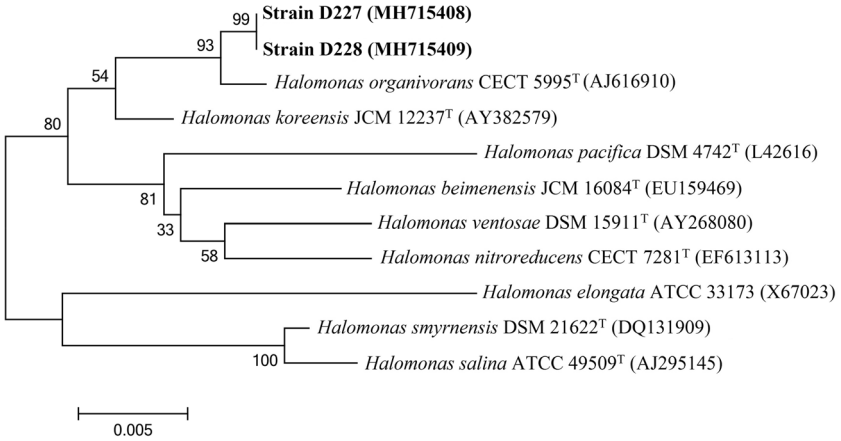

Fig. 1 Phylogenetic tree constructed using 16S rDNA gene sequences of the two selected strains belonging to the genus *Halomonas*. Bar, five subtitutions per 1000 nucleotides

content decreased at higher salt concentrations and only a small amount of ectoine (3.9 wt%) was detected in the presence of 27% NaCl (Fig. 4b). As shown in Fig. 4c, maximum ectoine content of 18.1 wt% (14.6 and 3.5 wt% ectoine and hydroxyectoine content, respectively) was achieved by strain D228 at 18% NaCl concentration. However, the total ectoine content dropped at higher salt concentrations and only ectoine was detected in the presence of 27% NaCl.

Effect of osmotic downshock on ectoine release rates and bacterial survival rates

The effect of osmotic downshock solutions containing different NaCl concentrations (0%, 2.5%, and 5%) on the amount of released ectoines and survival of the two selected bacterial strains was investigated. As shown in Fig. 5a, about 87% and 92% ectoines were released by strains D227 and D228, respectively, after 30 min when the bacterial cells (cultured in MPA medium containing 18% NaCl) were suspended in distilled water. The amount of released ectoines diminished to 64% and 61% for strain D227 and 86% and 76% for strain D228 when the NaCl concentration in the osmotic downshock solution was increased to 2.5% and 5%, respectively. In contrast, the bacterial survival rates increased from 6 to 86% and 98% for strain D227 and from less than 1 to 34% and 96% for strain D228 when the NaCl concentration in the osmotic downshock solution was increased from 0 to 2.5% and 5%, respectively (Fig. 5B).

Discussion

Two main strategies, namely, the "salt-in" and "compatible solute" strategies, are employed by microorganisms to cope with high external salt concentrations in the ecosystem that they inhabit. Accumulation of compatible solutes for osmotic adaptation is a typical mechanism found in many halotolerant and halophilic microorganisms (Roberts 2005). Compatible solutes include sugars, polyols, phosphodiesters, glyceric acid derivatives, and amino acids and their respective derivatives, betaines, and ectoine (Roberts 2005; Lentzen and Schwarz 2006). Ectoine is currently produced by "bacterial milking" process (Sauer and Galinski 1998), which involves repetitive cycles of two main steps: bacterial cells are first grown in hyperosmotic medium to induce ectoine accumulation and then harvested and transferred to osmotic downshock solution to promote ectoine release. Hence, bacteria that can grow and accumulate high amount of ectoine at a wide range of salt concentrations have promising application in this process.

It has been reported that the salt concentration in the soil water of the Can Gio mangrove forest is constantly below 3% (*w*/*v*) (Tuan and Kuenzer 2012; Costa-Böddeker et al. 2017). As a result, bacterial strains in the soil of mangrove forests are mainly halotolerant and moderately halophilic (Van-Thuoc et al. 2012). Hence, to find promising ectoine-producing bacteria, in the present study, soil samples collected from the Can Gio mangrove forest were incubated in MPA medium containing 15% NaCl for 5 days, which allowed the growth of halophilic or high salt-tolerant bacteria only. Among more than 200 halophilic and halotolerant bacterial colonies isolated, 12 strains were selected, all of which were able to produce ectoine (Table 1). The results of the present study showed that ectoine synthesis is a typical adaptation strategy that helps the isolated bacteria to survive under saline conditions in the mangrove ecosystem, suggesting that high ectoine-producing bacteria can be found in mangrove forests. A few previous studies found ectoine synthesis genes in the genome of some bacterial strains isolated from Malaysia such as *Microbulbifer* sp. CCM-MM1 (Moh et al. 2017), *Hahella* sp. strain CCB-MM4 (Sam et al. 2018), and *Streptomyces mangrovisoli* MUSC 149^T (Ser et al. 2018). However, there are no reports on the production of ectoine by bacteria isolated from mangrove forest.

Phylogenetic studies of the two selected ectoine-producing strains showed that they were the same species belonging to the genus *Halomonas* (Fig. 1). *Halomonas* is the biggest genus of the family *Halomonadaceae*, with more than 100 species reported so far (http://www.bacterio.net/halomonas.html). These bacteria are Gram-negative, motile, and rod-shaped and have been found in a wide variety of saline environments such as

Table 2 Phenotypic characteristics of the two selected strains and the reference strain *H. organivorans* (data obtained from García et al. 2004)

	D227	D228	*H. organivorans*
Morphological characteristics			
Shape	Rod	Rod	Rod
Size (μm)	0.5–0.7 × 1.1–3.0	0.5–0.8 × 1.0–2.8	1.0–1.2 × 2.0–3.0
Gram staining	–	–	–
Mobility	+	+	+
Spore formation	–	–	–
Growth conditions			
Optimum NaCl (%, *w/v*)	5–6	5–6	7.5–10
Optimum temperature (°C)	30–35	30–35	37
Optimum pH	6–7	6–7	7.0
Aerobic conditions	+	+	+
Biochemical characteristics			
Catalase	+	+	+
Ornithine decarboxylase	–	–	–
Arginine dihydrolase	–	–	NR
Lysine decarboxylase	–	–	NR
Urease	–	–	NR
L-Arabitol	–	–	NR
Galacturonate	–	–	+
5-Ketogluconate	+	+	NR
Lipase	–	–	NR
Phenol red	–	–	NR
Beta-glucosidase	+	+	NR
D-Mannitol	+	+	+
D-Maltose	+	+	–
Adonitol	+	+	–
Palatinose	+	+	NR
Beta-glucuronidase	–	–	NR
Manonate	–	–	NR
Indole formation	+	+	–
N-Acetyl-beta-glucosaminidase	–	–	NR
Beta-galactosidase	+	+	NR
D-Glucose	+	+	+
Sucrose	+	+	+
L-Arabinose	+	+	–
D-Arabitol	+	+	+
Alpha-glucosidase	+	+	NR

mangroves, seas, and saline lakes. Furthermore, *Halomonas* spp. are moderate halophiles, requiring salt concentrations between 3 and 15% (*w/v*) for growth (Mata et al. 2002; Gasperotti et al. 2018). Several *Halomonas* strains have been considered as ideal candidates for potential biotechnological applications, including degradation of organic pollutants and production of compatible solutes, biopolymer, and enzymes (Margesin and Schinner 2001).

In the present study, the 16S rDNA sequences of the two selected strains were 99.4% similar to that of *H. organivorans* (Fig. 1), with only some phenotypic differences (Table 2). *Halomonas organivorans* is a moderately halophilic bacterium that has been isolated from hypersaline habitats in southern Spain. This strain could utilize a wide range of organic compounds, such as benzoic acid, *p*-hydroxybenzoic acid, phenol, salicylic acid, cinnamic acid, and phenylpropionic acid, and can be employed for the decontamination of polluted saline habitats (García et al. 2004). However, there are no reports on the production of ectoine by *H. organivorans*, and the present study is the first to demonstrate ectoine accumulation in two *H. organivorans* strains from the Can Gio mangrove forest (Table 1).

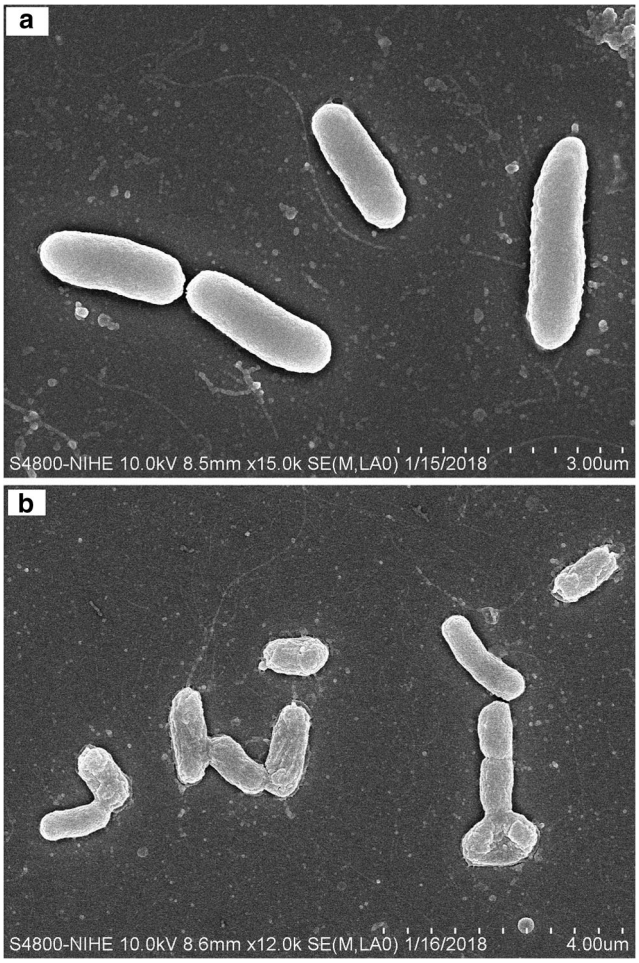

Fig. 2 Scanning electron micrographs of strains D227 (**a**) and D228 (**b**) showing their morphological features

Previous studies have established that the presence of NaCl is the key factor that significantly influences cell growth and ectoine accumulation of halophilic bacteria (Detkova and Boltyanskaya 2007; Van-Thuoc et al. 2010). With the main function of osmotic balance, the amount of ectoine in bacterial cells is

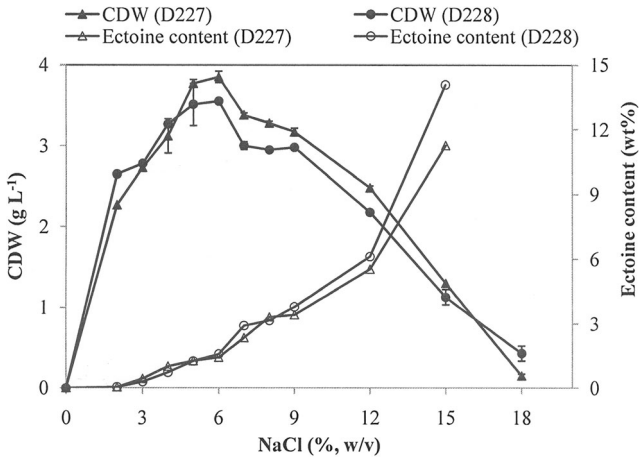

Fig. 3 Effect of NaCl concentration on cell growth and ectoine accumulation of the two selected strains

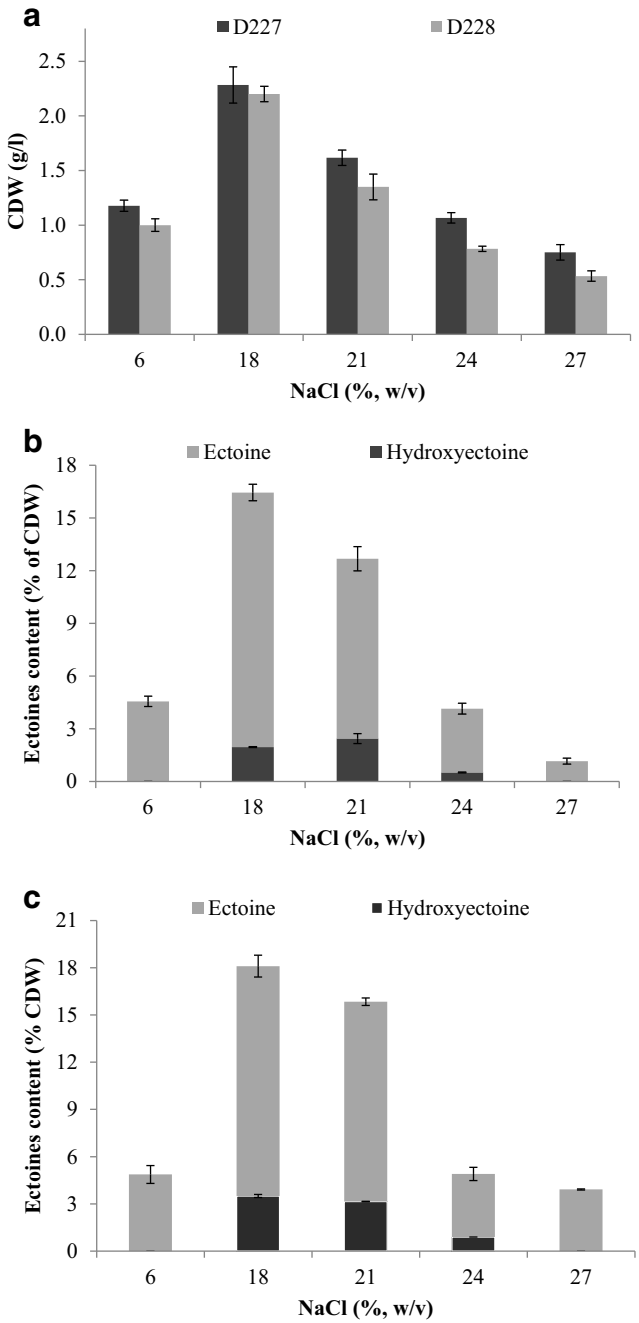

Fig. 4 Two-step fermentation for ectoine production by the two selected strains. The bacterial strains were first grown in MPA medium containing 6% NaCl, collected after 15 h of cultivation, and transferred to HM containing 18%, 21%, 24%, and 27% NaCl. CDW (**a**), ectoines produced by strain D227 (**b**), and ectoines produced by strain D228 (**c**) at different salt concentrations

controlled by the concentration of NaCl in the culture medium. In the present study, the ectoine content produced by the two isolated strains increased and reached the maximum value when the NaCl concentration in the culture medium was increased to 18% (Fig. 4b, c). The total ectoine content generated by strain D227 (16.5 wt%) and strain D228 (18.1 wt%) is comparable to that produced by commercial ectoine producers such as *H. elongata*

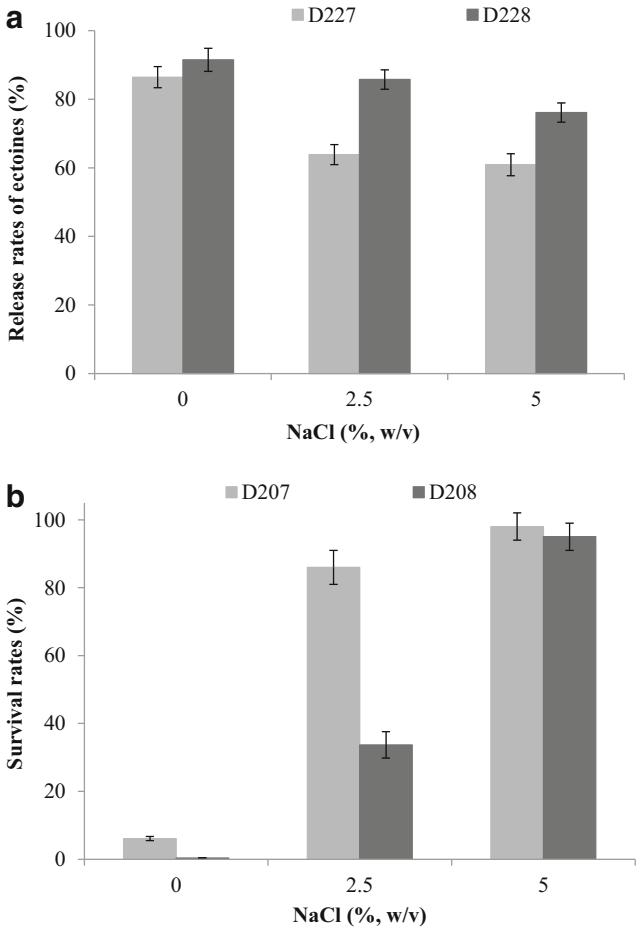

Fig. 5 Effect of NaCl concentration in the downshock solution on ectoine release rates (**a**) and survival rates (**b**) for the two selected strains

(15.5 wt%) (Sauer and Galinski 1998). Besides, a small amount of hydroxyectoine was also detected in the two selected strains when the NaCl concentration in the culture medium was 18% or higher (Fig. 4b, c), similar to that noted in earlier studies on other *Halomonas* spp. (Van-Thuoc et al. 2010; Öztürk et al. 2015). Hydroxyectoine is a better stress protector than ectoine and has been shown to preserve various biological functions (Lippert and Galinski 1992; Borges et al. 2002; Van-Thuoc et al. 2013). Halophilic bacteria tend to produce hydroxyectoine under severe stress conditions (Guzmán et al. 2009; Öztürk et al. 2015). Interestingly, in the present study, although the optimum NaCl concentration for the growth of the two selected strains was 3–6% (*w/v*), both the strains could grow at NaCl concentration of up to 21% (*w/v*) owing to protection from compatible solutes (ectoine and hydroxyectoine). The concentration of ectoine and hydroxyectoine in the two selected bacterial strains changed with the amount of NaCl in the culture medium, which is an important feature that helps the bacteria to adapt and survive under different environmental conditions. However, ectoine and hydroxyectoine could only help to protect the bacterial cells at certain concentrations of NaCl; the two selected strains were inhibited and destroyed when the NaCl concentration in the culture medium was higher than 21% (Fig. 4a).

Besides NaCl concentration, medium composition was also found to be an important factor affecting ectoine accumulation. At similar temperature and NaCl concentration, the MPA medium was noted to stimulate lower ectoine production than modified HM. Strains D227 and D228 produced 9.1 and 9.9 wt% ectoine, respectively, after 30 h of cultivation in MPA medium containing 15% NaCl (Table 1); in contrast, the ectoine content increased to 12.3 and 14.1 wt%, respectively, after 30 h of cultivation when HM containing the same concentration of NaCl was employed (Fig. 3). In general, halophilic and halotolerant organisms use a group of solutes (cocktails of solutes), and not just a single solute, for osmotic balance (Roberts 2005). The modified HM is a defined medium, while MPA is a complex medium containing precursors for various solutes. Thus, with the availability of these precursors in the culture medium, the bacterial cells tend to synthesize several molecules that together contribute to osmotic balance. Therefore, in addition to the salt concentration in the culture medium, regulation of the amount of precursors for ectoine and other compatible solutes in the culture medium is another approach to control ectoine accumulation in bacterial cells.

It is important to note that the ideal ectoine-producing strain must efficiently release the intracellularly accumulated ectoines when subjected to hypoosmotic shock and survive and resynthesize the osmolytes after downshock. Previous studies have demonstrated that the ectoine release rates and bacterial survival rates are controlled by the NaCl concentration in the downshock solution (Nagata et al. 2008; Van-Thuoc et al. 2013). While the lowest bacterial survival rates and highest ectoine release rates could be achieved by suspending bacterial cells in distilled water, an increase in the NaCl concentration in the downshock solution could lead to a decrease in ectoine release rates and an increase in bacterial survival rates (Nagata et al. 2008; Van-Thuoc et al. 2013). Similar result was also noted in the present study, with about 90% of accumulated ectoines released by the two bacterial strains in distilled water, whereas the release rates decreased when the NaCl concentration was increased to 2.5% and 5% (Fig. 5a). However, excess secretion of accumulated ectoines to the surrounding medium resulted in a decrease in the bacterial survival rates, with only 6% of strain D227 and less than 1% of strain 228 surviving after 30 min of incubation in distilled water (Fig. 5b). In contrast, the viability of cells increased in the presence of 2.5% and 5% NaCl. These results are comparable with those obtained with commercial strain (*H. elongata*). For instance, the ectoine release rates for strains D227 and D228 were 61% and 76%, respectively, while the

bacterial survival rates for the two strains were 98% and 96%, respectively, after 30 min of incubation in the presence of 5% NaCl. Meanwhile, the ectoine release rate and bacterial survival rate for the commercial strain *H. elongata* have been reported to be 64% and 86%, respectively, in the presence of 3% NaCl (Sauer and Galinski 1998).

In conclusion, the present study demonstrated that the two bacterial strains isolated from Can Gio mangrove soil can be promising candidates for commercial ectoine production. Both the strains were able to synthesize high contents of ectoine, rapidly release accumulated ectoine, and maintain high survival rates after being subjected to hypoosmotic shock. Currently, studies on the optimization of the culture medium for ectoine production using the two isolated strains are in progress.

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Compliance with ethical standards

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

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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