

Ectoine and Hydroxyectoine from Halophilic Bacteria Isolated from Traditional Solar Saltern at Pejarakan Village, Buleleng Regency, Bali

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ABSTRACT

The increased use of ectoine and hydroxyectoine in cosmetics and pharmaceuticals has led to the increasing demand for these bioactive compounds. This study aims to investigate the production of ectoine and hydroxyectoine by halophilic bacteria isolated from traditional solar saltern at Pejarakan Village, Buleleng Regency, Province of Bali, Indonesia. The halophilic bacteria were grown from the brine and the sediment samples using Luria Bertani media. The production of ectoine and hydroxyectoine was determined by inoculating the bacteria in MM63 media containing 12% w/v NaCl. Ectoine and hydroxyectoine were then extracted from the bacterial cells using a mixture of methanol/chloroform/water (10/5/4). Finally, the ability of the bacteria to excrete ectoine and hydroxyectoine was investigated using the osmotic downshock technique. The results showed that a total of 88 halophilic bacteria have been isolated which showed varied salt tolerances from 0 to 27.5% w/v. Most of the bacteria (86 isolates) were able to grow in MM63 media, suggesting the potential of the bacteria producing ectoine and hydroxyectoine. Further investigation showed that 33 selected halophilic bacteria were able to produce ectoine and hydroxyectoine with levels of 9.1 to 301.8 mg/L and 3.9 to 122.1 mg/L, respectively. All the bacteria were also able to excrete ectoine and hydroxyectoine after the osmotic downshock process with levels of 9.8 to 277.5 mg/L and 14.3 to 122.7 mg/L, respectively. The best ectoine and hydroxyectoine-producing bacteria showed the closest homology relationship with *Salinivibrio costicola* and *Salinivibrio kushneri*. Our study has found several promising candidates for ectoine and hydroxyectoine producers.

1. Introduction

Ectoine and hydroxyectoine are important raw materials for pharmaceuticals and cosmetics. Ectoine is used in various formulas such as lozenges in the treatment of acute viral pharyngitis (Dao *et al.* 2019), mouthwash for oral mucositis chemotherapy (Dao *et al.* 2018), skincare and sunscreens (Hseu *et al.* 2020), and adjuvant for the treatment of allergic rhinitis and rhinosinusitis and eye drops (Casale *et al.* 2019; Werkhauser *et al.* 2014). Some pharmaceutical products and cosmetics use ectoine such as ectoin dermatitis cream, ectoin allergy nasal spray, ectoin mouth, and throat spray, ectoine rhinitis nasal spray, revitalizing face cream, and ectoine sicca eye drops (Becker and Wittmann 2020).

The increased production and diversification of pharmaceutical and cosmetic products have

led to increasing demand for ectoine with a global consumption rate of 15,000 tons per year (Goraj *et al.* 2019). Up to the present, the world's ectoine supply is dominated by Bitop AG, one of the largest ectoine companies in Germany. The company used *Halomonas elongata* as an ectoine producer. Although the productivity of the bacteria producing ectoine was significantly high (10 g/L culture), the inefficient production process caused the production capacity of ectoine to be unable to meet the increasing market demand. Therefore, the search for an effective and efficient formula for ectoine production is essential. One of the efforts is finding an efficient and effective ectoine-producing bacteria.

Ectoine and hydroxyectoine are secondary metabolites produced by halophilic microorganisms as a protection strategy of the cell against high salt levels in their habitat. Indonesia has various types of ecosystems with high salinity, including salt ponds (Wei *et al.* 2011), mangrove muds (Van-Thuoc *et al.* 2019), and shallow and deep-sea sediments

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(Anburajan *et al.* 2019). However, research exploring the potential of halophilic bacteria from these ecosystems is still very limited.

Bali is one of exotic islands in Indonesia which has various habitats with extreme salinity where the potential of halophilic microorganisms has not been mapped and systematically researched. One of the potential halophilic habitats is the traditional solar saltern at Pejarakan Village, Buleleng Regency, Bali Province, Indonesia. Solar saltern is one of the potential artificial habitats for halophilic bacteria. The gradual concentrating of seawater to be salt crystals in solar saltern causes changes in salinity that trigger microbial adaptation mechanisms to different salt levels. This newly formed habitat stimulates the microbial metabolic system and triggers changes in the production of secondary metabolites (Conde-Martinez *et al.* 2017). Considering the potential of this solar saltern, this study focuses on exploring a new ectoine and hydroxyectoine-producing bacteria from the solar saltern of Pejarakan Village.

2. Materials and Methods

2.1. Chemicals and Media

All chemicals such as yeast extract, tryptone, KH_2PO_4 , KOH, $(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, Glucose $\cdot\text{H}_2\text{O}$, NaCl, NaOH, HCl, and solvents such as methanol, chloroform, and acetonitrile were purchased from Merck Germany with pro analysis grade. High purity of ectoine and hydroxyectoine was purchased from Sigma Aldrich. Bacteria were grown in Luria Bertani (LB) media composed of (per liter): 5 g tryptone, 5 g yeast extract, 20 g bacto agar, and 100 g NaCl. Ectoine production was generated using MM63 media with the composition of (per liter): 13.61 g KH_2PO_4 , 4.21 g KOH, 1.98 g $(\text{NH}_4)_2\text{SO}_4$, 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0011 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g Glucose $\cdot\text{H}_2\text{O}$, and varying concentrations of NaCl. pH was adjusted to 7.1 using HCl and NaOH (Fatollahi *et al.* 2020).

2.2. Isolation and Determination of the Salt Tolerance of the Halophilic Bacteria

The halophilic bacteria were grown from the brine and sediment samples obtained from the solar saltern of Pejarakan Village. The sediments were diluted in sterile water containing 10% w/v NaCl, and then filtered. The sediment filtrate and the brine sample were spread on solid Luria Bertani media containing 10% w/v NaCl and incubated at 37°C for 2 to 4 days. Each bacterial colony was then isolated to obtain a pure isolate of each bacteria. The bacterial colonies were streaked on solid LB media and incubated at 37°C

for 1 to 2 days. The steps were repeated two to three times until uniform bacterial colonies were obtained.

The salt tolerances of the halophilic bacteria were determined using solid Luria Bertani media containing varying levels of NaCl, i.e. 0 to 27.5% w/v. Each bacteria was grown on the media and incubated at 37°C for 1 to 3 days. The salt tolerances of the bacteria were then determined by observing the growth level of each bacteria on the media.

2.3. Potential Test of Ectoine and Hydroxyectoine-Producing Bacteria

The potentials of the halophilic bacteria to produce ectoine and hydroxyectoine were investigated using solid MM63 media containing various levels of NaCl, i.e. 10%, 15%, and 20% w/v. The bacteria were grown on the media at 37°C for 2 to 4 days. The ectoine-producing bacteria was determined by observing the growth level of each bacteria on the media.

2.4. Production of Ectoine and Hydroxyectoine from the Halophilic Bacteria

Initially, the bacteria were inoculated in 5 ml of MM63 broth containing 12% w/v NaCl in a shaker for 20 hours at 37°C. Subsequently, 1 ml of inoculum was transferred aseptically into 25 ml of fresh MM63 media. The bacteria were incubated in a shaker at 37°C with a speed of 170 rpm for 48 hours. The bacterial cells were then separated by centrifugation at 8,000 x g for 10 min to determine the ectoine and hydroxyectoine level and cell density.

The cell density was determined following the procedure proposed by Van-Thuoc *et al.* (2010). The cell pellet was washed with phosphate buffer pH 7.1 containing NaCl at the same level as the previous medium. After centrifugation, the cells were then dried in an oven at a temperature of 70°C to obtain a constant weight. The cell density is expressed as a gram of cell dry weight (cdw) per liter of inoculum.

2.5. Release of Ectoine and Hydroxyectoine by the Bacteria using the Osmotic Downshock Technique

Bacterial cells were cultured in MM63 broth containing 12% w/v NaCl in a shaker at 37°C with agitation speed of 170 rpm for 48 hours to produce ectoine and hydroxyectoine. The cells were then separated by centrifugation at 8,000 x g for 10 min and subsequently transferred aseptically into 1% w/v NaCl containing distilled. The bacteria were then shaken for 30 minutes at room temperature and agitation speed of 150 rpm to release ectoine

and hydroxyectoine. Ectoine and hydroxyectoine were then separated from the bacterial cells by centrifugation at 8,000 x g for 10 minutes and determined using high performance liquid chromatography (HPLC).

2.6. Extraction and Determination of Ectoine and Hydroxyectoine

Ectoine and hydroxyectoine were extracted following Bligh and Dyer procedure (Van-Thuoc *et al.* 2019). The cell pellets were dried at 70°C for 1 hour, then extracted with 350 µl of methanol/chloroform/water (10:5:4, vol/vol/vol). The extraction process was performed using ultrasonication for 50 minutes. An equal volume (65 µl) of chloroform and water were then added. The mixture was shaken for 10 min. Phase separation was enhanced by centrifugation at 10,000 x g for 15 min. The water phase containing ectoine and hydroxyectoine was recovered for HPLC analysis.

Twenty microliters of each sample were analyzed on a Silica C18 column (Sigma Aldrich, USA). Ectoine and hydroxyectoine were monitored by their absorbance at 210 nm using a UV/VIS detector. The solvent employed for compatible solute separation was water/acetonitrile (95/5). Chromatography was carried out isocratically at a flow rate of 1 ml/min and 33°C using Shimadzu high-performance liquid chromatography. Ectoine and hydroxyectoine were determined using authentic ectoine and hydroxyectoine purchased from Sigma Aldrich. The structure of ectoine was confirmed by ¹H-NMR analysis using A500a Agilent DD2 500 MHz NMR spectrometer.

2.7. Identification of the Halophilic Bacteria

The bacteria were identified based on their 16S rRNA gene. Chromosomal DNA of the bacteria was isolated using Presto Mini gDNA Bacteria Kit (Geneaid). The 16S rRNA gene was then amplified using the polymerase chain reaction (PCR) using primer Bact27F (order: AGAGTTTGATCATGGCTCAG) and Uni1492R (order: GGTTACCTTGTTACGACTT). The reaction was carried out following steps: predenaturation (94°C for 4 min), denaturation (94°C for 30 seconds), annealing (50°C for 1 min), and extension/propagation (72°C for 2 min). The cycle was repeated 34 times. The gene of 16S rRNA was then sequenced and analyzed using software DNA Baser version 3.5.4 (Heracle BioSoft). The acquired sequence was then aligned using BLAST (basic local alignment sequence tool). The sequence and related sequences obtained were further analyzed to construct the phylogenetic tree using

MEGA (molecular evolutionary genetics analysis) version 5.0. The Gram type and morphology of the bacterial cell were observed using an Olympus CX-21 microscope with magnification of 100x.

3. Results

3.1. The Salt Tolerance of the Halophilic Bacteria

A total of 88 halophilic bacteria were successfully isolated from the brine and the sediment samples obtained at the solar saltern located at Pejarakan Village, Buleleng Regency, Province of Bali, Indonesia. The bacteria showed diverse growth on LB media containing varied levels of NaCl from 0% to 27.5% w/v. Based on their salt tolerance, the halophilic bacteria were then grouped into seven groups (Table 1). Most of the bacteria showed a wide range of salt tolerance from 0.5% to 20% w/v and from 0.5% to 22.5% w/v, i.e., bacterial groups II and III, respectively. Several halophilic bacteria grew at an extreme salt level up to 25% (groups V and VII) and up to 27.5% w/v (group VI).

Interestingly, several bacteria in group I showed significant growth in a medium without the addition of salt. However, several halophilic bacteria need a minimum level of NaCl of 5% w/v to support their observable growth, i.e. isolate K20 (5). In addition, a minimum salt level of 2.5% w/v was required by the bacterial group of IV, V, and VI to reach sufficient growth.

3.2. The Potential of the Halophilic Bacteria Producing Ectoine and Hydroxyectoine

The potentials of the halophilic bacteria in producing ectoine and hydroxyectoine were investigated by observing the bacterial growth on solid MM63 media containing varying levels of NaCl, i.e., 10%, 15%, and 20% w/v. A total of 86 halophilic bacteria showed observable growth in MM63 media. Based on their salt tolerance in MM63 media, the bacteria were grouped into four groups (Table 2). Most of the bacteria, i.e., group I showed significant growth in MM63 media containing 10% w/v NaCl. Meanwhile, one isolate showed a comparable growth in the media containing 15% w/v NaCl, i.e., isolates K20 (5). Several halophilic bacteria in group III grew in MM63 media containing NaCl level 10% and 15% w/v. In addition, several halophilic bacteria showed an observable growth in MM63 media containing a wide range of NaCl levels of 10% to 20% w/v, i.e., group IV which consists of isolates K20 (2), K20 (4), K20 (12), K20 (15), and K20 (16).

Table 1. The salt tolerance of the halophilic bacteria isolated from the solar saltern of Pejarakan Village

Group	Bacterial isolate	Salt tolerance (% w/v NaCl)
I	K10 (6); K10 (20,21,31)	0-20
II	K10(1,2,3,7,8,12,13,14,15,16,17,18,19,27,28,29,32,33,34,35,36,37,38,39,40,41,42,43,44,46, 49) ; K10 (4,9,10,11,22,24,25,26,52)	0.5-20
III	K15 (1,2,3,4,5,6,7,8,9,10,11,12,13,14,1,16,17,18,19,20)	0.5-22.5
IV	K10 (5,45,47,48,50)	2.5-20
V	K10 (23,30,51)	2.5-25
VI	K20 (1,2,3,4,6,7,8,9,10,11,12,13,14,15,16)	2.5-27.5
VII	K20 (5)	5-25

Table 2. Group of halophilic bacteria based on their salt tolerance in MM63 media

Group	Bacterial isolate	Salt tolerance (% w/v NaCl)
I	K10 (1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 21, 23, 24, 25, 26, 27, 28, 29, 31, 32, 33, 34, 35, 36, 37, 39, 40, 41, 42, 46, 48, 49, 50 51, 52); K15 (1, 3, 4, 6, 7, 8, 9, 12, 14, 15, 17, 18, 19, 20)	10
II	K20 (5)	15
III	K10 (19, 20, 22, 30, 38, 44, 45, 47); K15 (2, 5, 10, 11, 13, 16) K20 (1, 3, 6, 7, 8, 9, 10, 11, 13, 14)	10-15
IV	K20 (2,4,12,15,16)	10-20

3.3. Ectoine and Hydroxyectoine Produced by the Halophilic Bacteria

A total of 33 halophilic bacteria were selected to investigate the level of ectoine produced intracellularly and the level of ectoine excreted by the halophilic bacteria after the osmotic downshock process. The ectoine and hydroxyectoine produced by the bacteria were confirmed by HPLC chromatogram using authentic ectoine and hydroxyectoine, as shown in Figure 1. The ectoine and hydroxyectoine were proved by a peak at the retention time of 3.05 and 2.92 minutes, respectively. The ¹H-NMR spectrum further confirmed the molecular structure of ectoine. The ¹H-NMR spectrum of ectoine from halophilic bacteria shows proton signals at chemical shifts similar to authentic ectoine (Figure 2). The proton multiplet appears at a chemical shift (δ) of about 2.1 ppm corresponding to the methylene proton (-CH₂-) present in the CH₂-CH = N group. At a chemical shift of about 2.25 ppm, a singlet signal indicates the presence of a methyl proton (-CH₃) in the CH₃-C = N group. The multiplet signal at a chemical shift of about 3.3 ppm indicates the presence of a methylene proton (-CH₂-) of the -NH-CH₂ group. Meanwhile, the triplet signal that appears at a chemical shift of about 4.1 ppm indicates the presence of a methine proton (-CH-) from the N-CH-CO₂H group.

The concentrations of intracellular ectoine produced by the halophilic bacteria varied widely, ranging from 9.1 to 301.8 mg/L (Figure 3 to 5). The bacteria from colonies K20 showed lower concentrations of ectoine production (below 100 mg/L), hence less potential to be developed as ectoine producers (Figure 3). Meanwhile, the concentrations of intracellular ectoine produced by the bacteria of K15 colonies were in the range of 100 mg/L but still below 140 mg/L (Figure 4). The halophilic bacteria from the K10 colonies showed wider concentrations of ectoine production ranging from 63.3 to 301.8 mg/L (Figure 5). Several bacteria of these colonies, i.e., K10 (3), K10 (5), and K10 (21), were able to produce the highest concentrations of ectoine of 263.4, 201.4, and 301.8 mg/L, respectively.

All of the 33 halophilic bacteria tested were able to excrete their ectoine after the osmotic down shock process using distilled water containing 1% w/v NaCl. The concentrations of excreted ectoine varied widely from 9.8 to 277.5 mg/L (Figure 3 to 5). The bacteria from K20 colonies showed lower concentrations of excreted ectoine with a value of 9.8 to a maximum of 87 mg/L (Figure 3). Meanwhile, K15 colonies could excrete their ectoine with concentrations ranging from 82.9 to 133 mg/L (Figure 4). Interestingly, the bacteria of K10 colonies showed higher excreted ectoine with concentrations

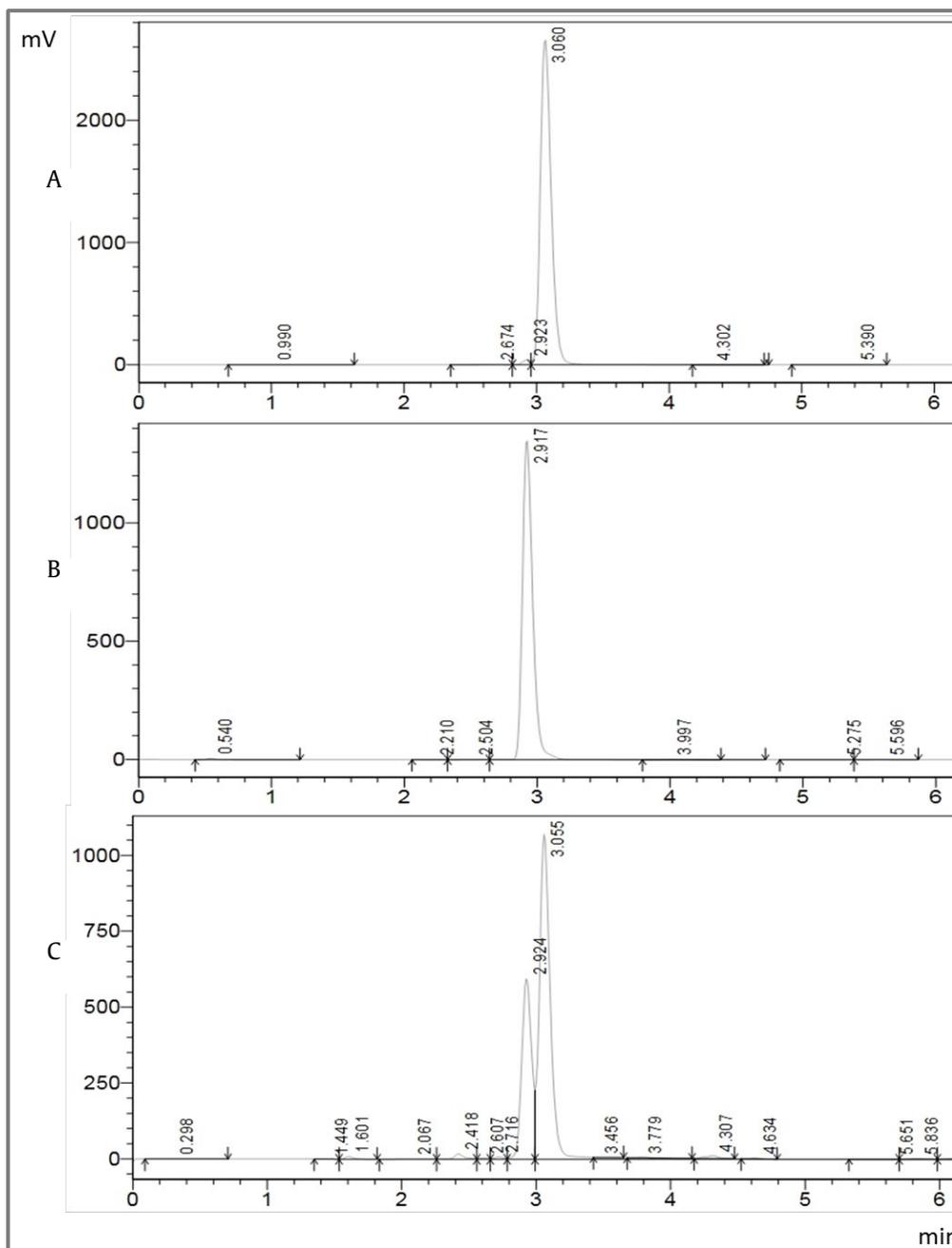


Figure 1. HPLC chromatograms of ectoine and hydroxyectoine. (A) authentic ectoine, (B) authentic hydroxyectoine, (C) ectoine, and hydroxyectoine extracted from the halophilic bacteria

ranging from 58.8 to 277.5 mg/L (Figure 5). Several of them, i.e., K10 (3), K10 (5), K10 (21), and K10 (23) were able to excrete ectoine with concentrations above 150 mg/L, i.e., 156.3, 172.1, 277.5, and 158.1 mg/L, respectively.

All halophilic bacteria tested were also able to produce hydroxyectoine with concentrations lower than ectoine, as shown in Figures 3 to 5. An average ratio of hydroxyectoine to ectoine produced by the halophilic bacteria was 1 to 2 or around 50%.

Generally, the level of hydroxyectoine was in line with the level of ectoine produced by the bacteria. The bacteria that produced a higher level of ectoine were also able to produce a higher level of hydroxyectoine. For those, the bacterial colonies K20 were the lowest hydroxyectoine producer with concentrations of 10.5 to 28.2 mg/L (Figure 3). Meanwhile, the concentrations of hydroxyectoine produced by the bacteria K15 were higher than K20, i.e., up to 68.5 mg/L (Figure 4). The bacterial colonies K10 were the highest hydroxyectoine

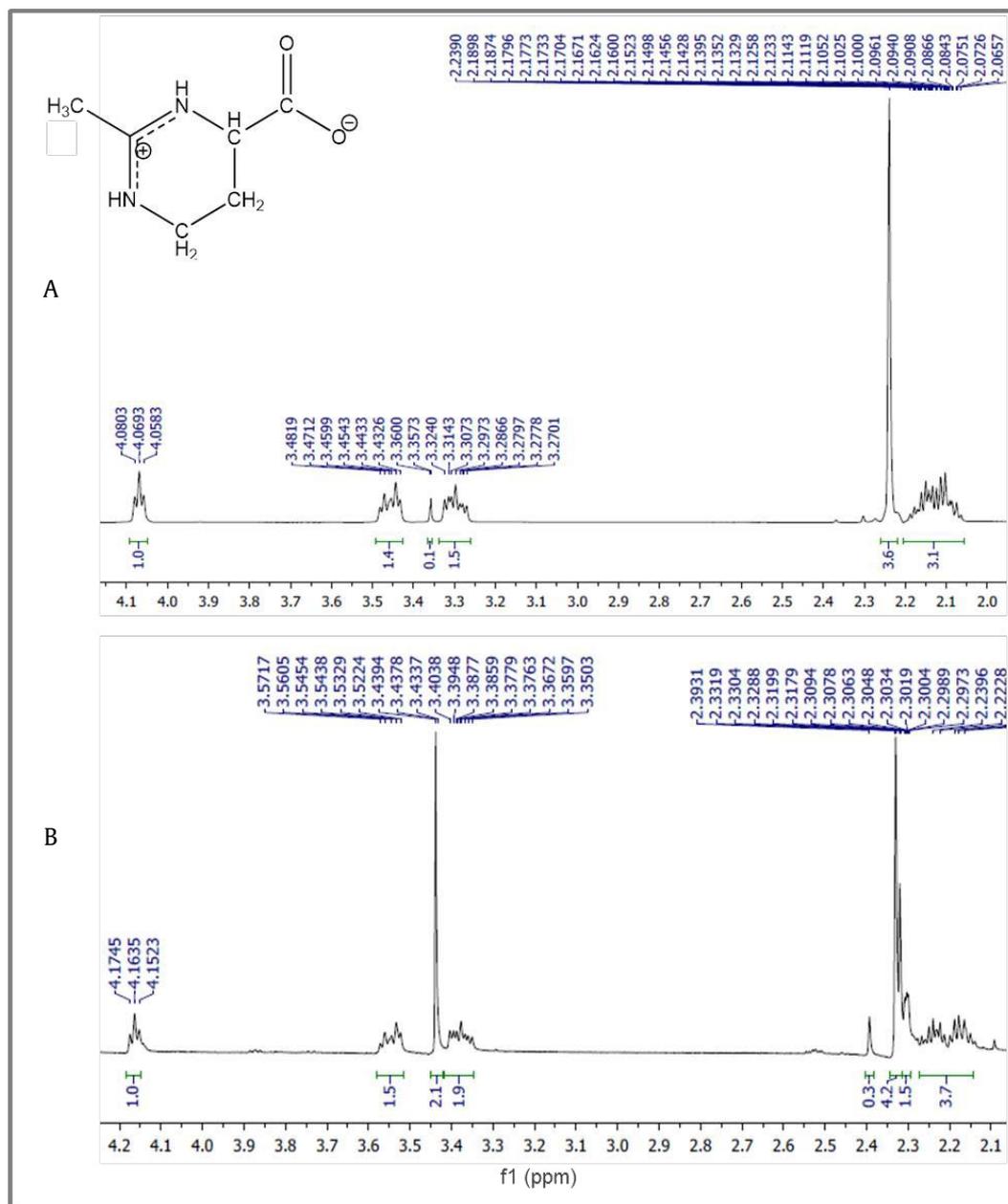


Figure 2. $^1\text{H-NMR}$ spectrums of ectoine. (A) authentic ectoine and (B) ectoine extracted from the halophilic bacteria

producer with concentrations up to 122.1 mg/L (Figure 5). Three bacteria of these colonies, i.e., K10 (3), K10 (5), and K10 (21), were able to produce the highest concentrations of hydroxyectoine of 104.3, 101.3, and 122.1 mg/L, respectively.

The halophilic bacteria were also able to excrete hydroxyectoine after the osmotic downshock treatment. The excreted hydroxyectoine concentrations varied widely from 14.3 to 122.7 mg/L (Figure 3 to 5). The K20 colonies showed the lowest concentrations of excreted hydroxyectoine followed by the colonies of K15 with maximum levels of 32.7 and 78.4 mg/L,

respectively (Figure 3 and 4). Meanwhile, the bacteria of K10 colonies were able to excrete up to 122.7 mg/L of their hydroxyectoine (Figure 5).

3.4. Identity of the Best Ectoine and Hydroxyectoine-producing Bacteria

The microscopic observation using Gram staining showed that all of the best ectoine-producing bacteria, i.e., K10 (3), K10 (5), and K10 (21), belonged to the Gram-negative bacteria with cocci cell shape (Figure 6). Based on the 16S rRNA gene sequence, the

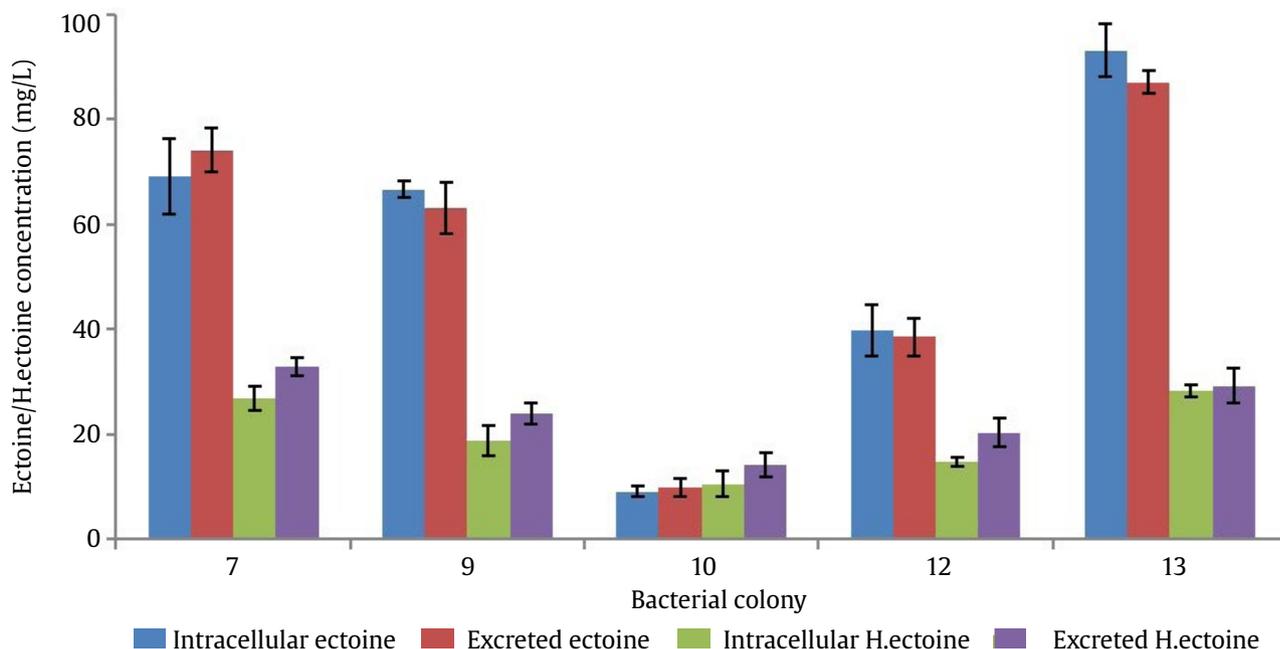


Figure 3. Production and excretion of ectoine and hydroxyectoine by the halophilic bacteria from colony K20

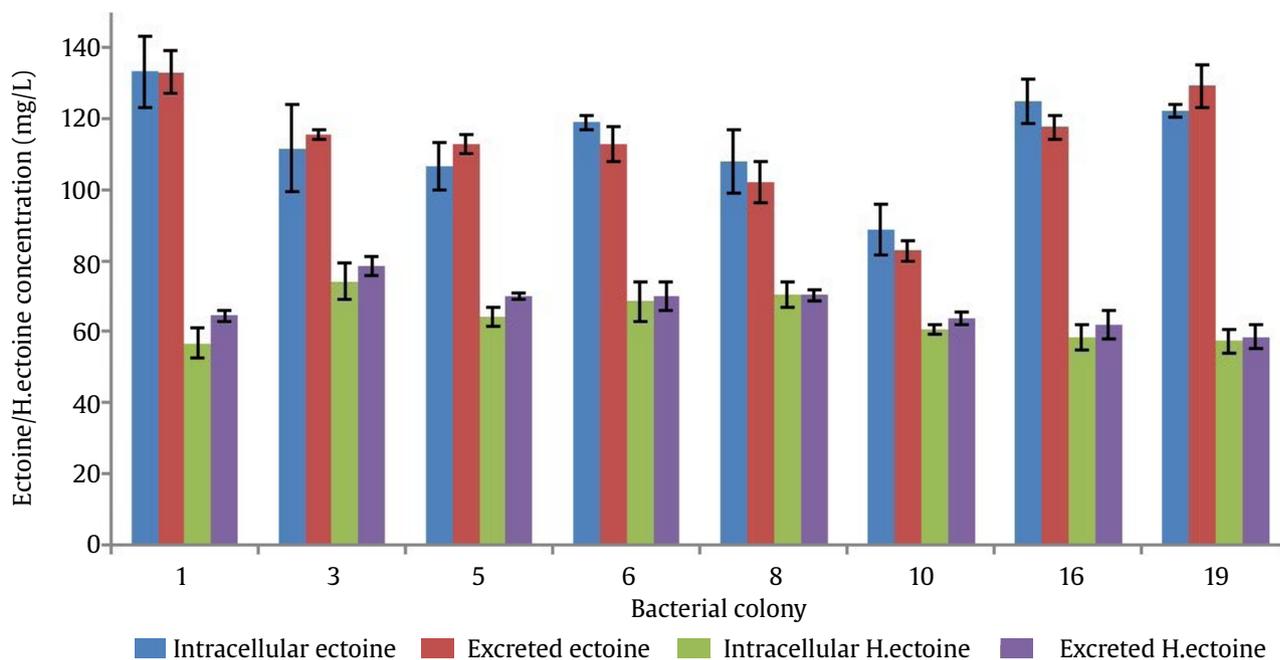


Figure 4. Production and excretion of ectoine and hydroxyectoine by the halophilic bacteria from colony K15

best ectoine and hydroxyectoine-producing bacteria were identified, as shown in Figure 7. The neighbor-joining phylogenetic trees revealed that the bacteria showed the closest homology relationship with *Salinivibrio costicola* for isolates K10 (3) and K10 (21) and *Salinivibrio kushneri* for isolate K10 (5).

4. Discussion

A total of 86 halophilic bacteria isolated from the solar saltern of Pejarakan Village showed diverse salt tolerance from 0% to 27.5% w/v (Table 1), indicating that the bacteria were able to respond

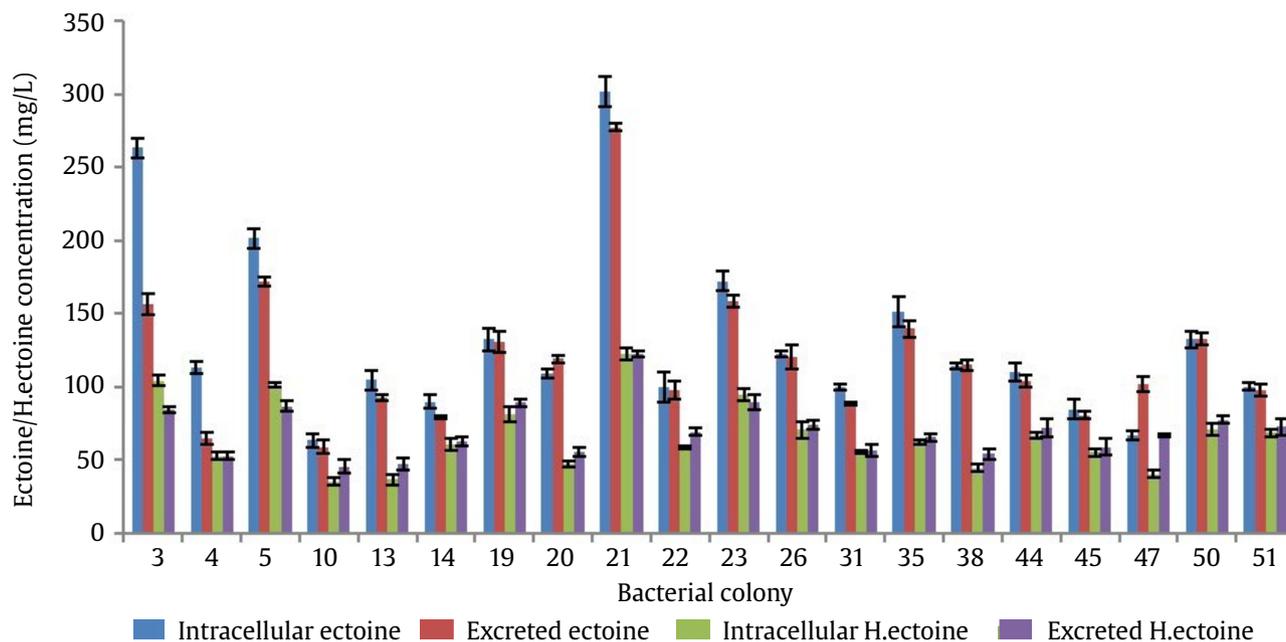


Figure 5. Production and excretion of ectoine and hydroxyectoine by the halophilic bacteria from colony K10

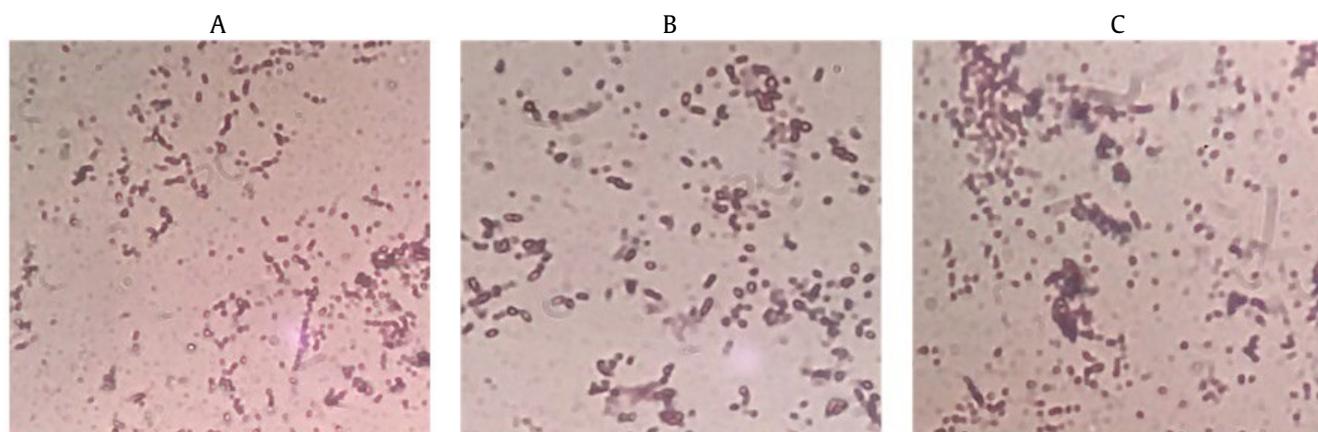


Figure 6. Gram staining and cell morphology observation results of the best ectoine and hydroxyectoine-producing bacteria. (A) colony K10 (3), (B) colony K10 (5), and (C) colony K10 (21)

to a wide range of salt level changes. This character is beneficial for the synthesis and excretion of ectoine and hydroxyectoine. In addition, the bacteria showed observable growth in MM63 media (Table 2), suggesting that the bacteria were able to produce ectoine and hydroxyectoine. The diverse growth of the halophilic bacteria in MM63 media containing the varied salt levels indicates the diverse characteristic of the ectoine-producing potential of the bacteria. The subsequent test of a total of 33 selected halophilic bacteria has proved the ability of the bacteria to produce ectoine and hydroxyectoine with concentrations up to 301.8 mg/L and 122.1 mg/L, respectively (Figure 3 to 5). This finding is

promising to obtain a new potential bacterial strain as an effective and efficient ectoine producer.

Ectoine or hydroxyectoine is synthesized by bacteria intracellularly so that an increase in biomass production increases ectoine levels. Therefore, the level of ectoine or hydroxyectoine is directly proportional to the level of biomass produced by the bacteria. The relationship between the cell dry weight (cdw) and the ectoine and hydroxyectoine produced by the halophilic bacteria is shown in Figure 8. The levels of cdw produced by the K20 colonies were below 3.5 mg/ml. Hence, the ectoine levels produced were also relatively low. Meanwhile, bacteria from the K15 colony were able to produce higher cdw of

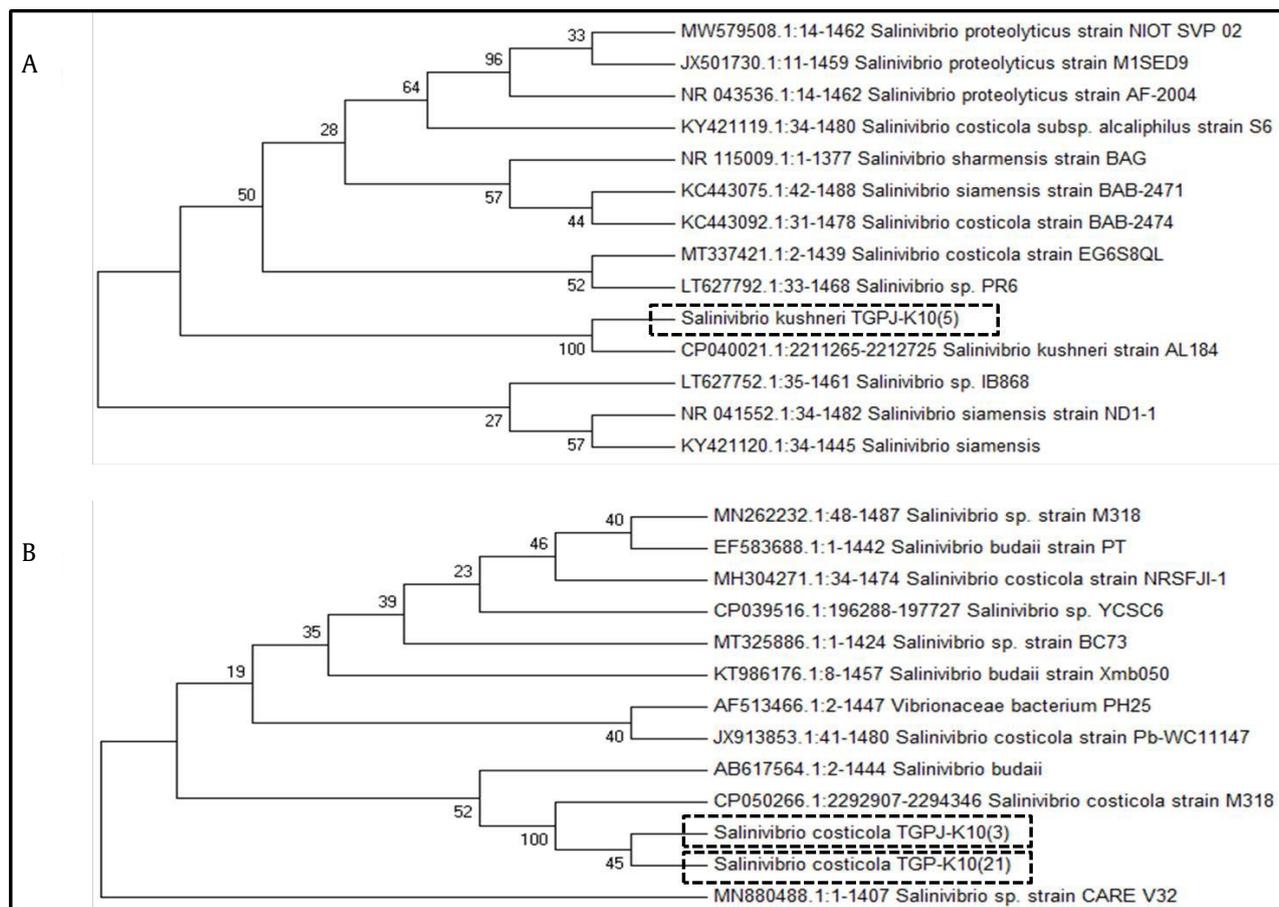


Figure 7. Neighbour-joining phylogenetic trees of the best ectoine and hydroxyectoine-producing bacteria. (A) colony K10 (5), (B) colonies K10 (3), and K10 (21)

up to 6.35 mg/mL so that the ectoine produced was higher than the K20 colony. In addition, K10 colonies showed a very diverse cdw production ranging from 2 to 6.7 mg/ml. Thus, the ectoine levels were also varied. However, several halophilic bacteria with low biomass production were able to produce high levels of ectoine. For example, the bacterial colonies of K10 (3) and K10 (5) that produced cdw levels of 3.0 and 2.85 mg/ml, respectively, were able to produce ectoine with the levels of 263.4 and 201.4 mg/L, respectively. Meanwhile, bacteria K15 (10), with a biomass production of 6.35 mg/ml, could only produce ectoine with a level of 88.8 mg/L. These results indicate that the capacity of ectoine synthesis in the cells of each bacteria varies.

As a derivative molecule of ectoine, the level of hydroxyectoine is also directly proportional to the biomass level produced by the bacteria (Figure 8). The bacteria with higher levels of biomass generally produced more hydroxyectoine. However, several halophilic bacteria with lower biomass production showed higher levels of hydroxyectoine production. For example, the bacterial colonies of K10 (3) and

K10 (5) with cdw levels of 3.0 and 2.85 mg/ml, respectively, were able to produce hydroxyectoine at 104.3 and 101.3 mg/L, respectively. Meanwhile, bacteria K10 (35) that produced 6.7 mg/ml biomass could only produce 62.4 mg/L of hydroxyectoine. These findings indicate that the capacity of hydroxyectoine production of each bacteria also varies.

The halophilic bacteria in this study could excrete more than 50% of ectoine produced (Figure 9). Only two colonies, namely K10 (3) and K10 (4) excreted less than 60% of their ectoine, while the other colonies could excrete more than 85% of the ectoine produced. Several colonies were even able to excrete ectoine more than 100%, i.e. K10 (20, 38, 47, 50), K15 (3, 5, 19), and K20 (7 and 10). Interestingly, the bacterial colony K10 (47) showed an ability to excrete up to 152.4% of its ectoine. This is possibly due to the ability of the bacteria to synthesize ectoine during the osmotic downshock process in response to the ectoine released by the cell.

The percentage of excreted hydroxyectoine was higher than that of excreted ectoine (Figure 9). The

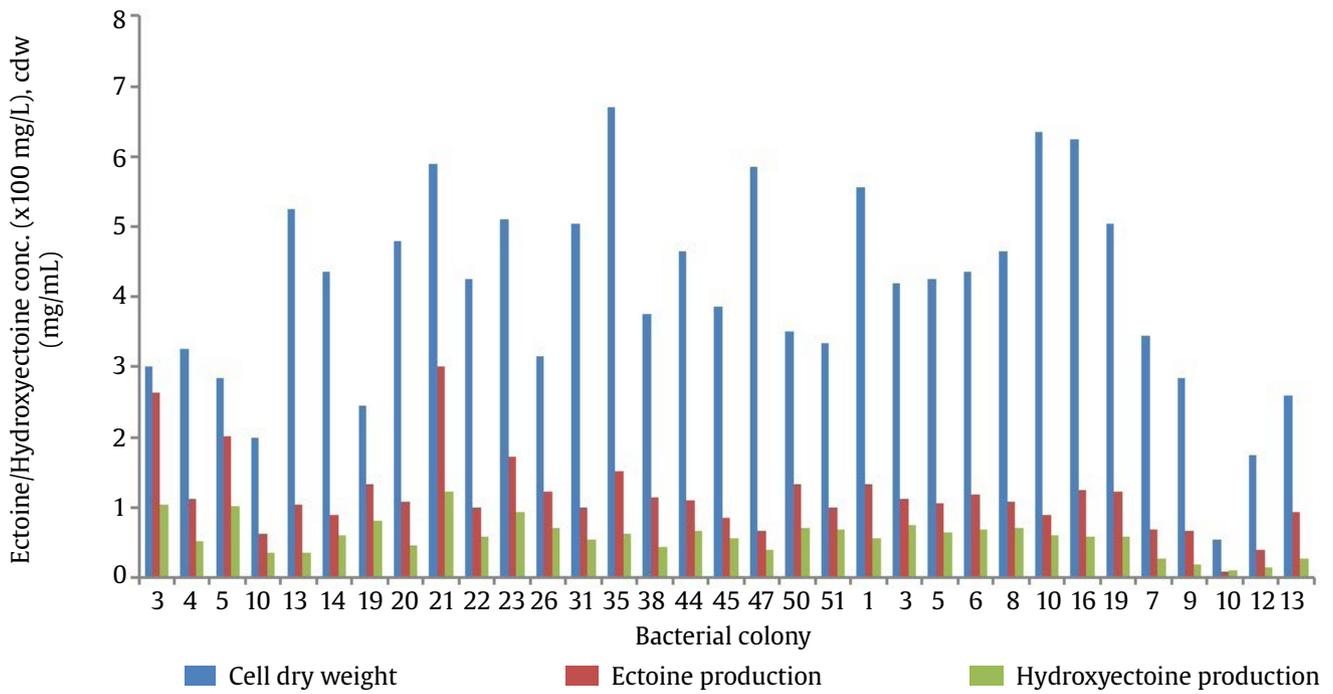


Figure 8. The relationship between the cell dry weight (cdw) and the concentration of ectoine and hydroxyectoine produced by the halophilic bacteria. Bacterial colony order: K10 (3 to 51), K15 (1 to 19), and K20 (7 to 13)

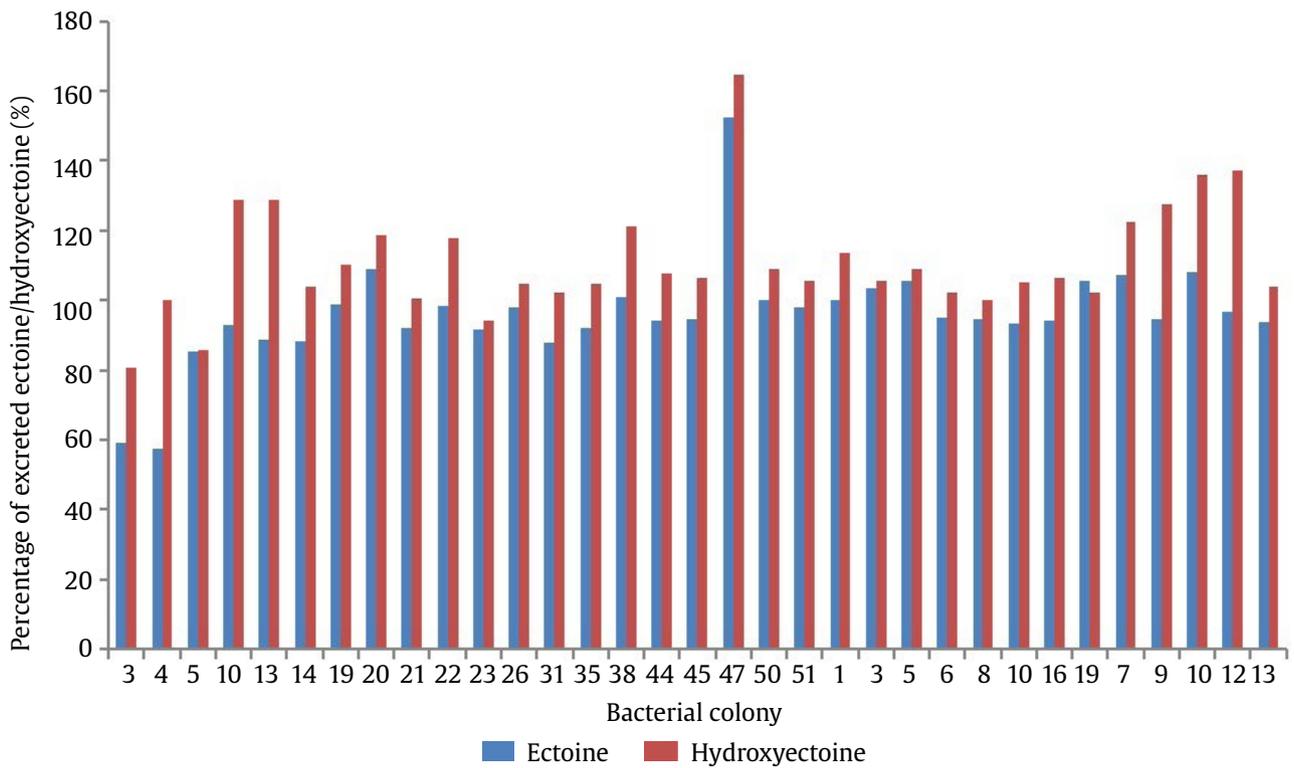


Figure 9. The percentage of excreted ectoine and hydroxyectoine by the halophilic bacteria after the osmotic downshock process. Bacterial colony order: K10 (3 to 51), K15 (1 to 19), and K20 (7 to 13)

halophilic bacteria in this study could excrete more than 80% of their hydroxyectoine and reached the maximum percentage of 164.8%. Only four bacteria from colonies of K10 (3, 5, 23) and K15 (8) excreted their hydroxyectoine less than 100%, while the other colonies were more than 100%. Interestingly, two bacteria, i.e., K10 (47) and K20 (12), showed an ability to excrete hydroxyectoine with the highest percentage of 164.8% and 137.3%, respectively. However, the level of excreted hydroxyectoine by these bacteria was still low at about 66.7 and 20.3 mg/L, respectively.

Recently, studies on the explorations of ectoine and hydroxyectoine-producing bacteria have been reported. Van-Thuoc *et al.* (2019) have investigated the ectoine-producing bacteria from Can Gio mangrove soil samples in Vietnam. The results showed that two strains among more than 200 isolates obtained, i.e., D227 and D228, could produce ectoine with levels of 0.11 and 0.12 g/L, respectively, after incubated for 30 hours in meat peptone agar (MPA) containing 15% NaCl. Both strains D227 and D228 were identified as *Halomonas organivorans*. Meanwhile, the levels of ectoine produced by the best ectoine-producing halophilic bacteria in our study were higher. Three strains of our bacteria, i.e., K10 (3), K10 (5), and K10 (21), were able to produce 0.263, 0.201, and 0.302 g/L ectoine, respectively after incubated for 28 hours in MM63 media containing 12% w/v NaCl. The bacteria were also reported able to release 61% and 76% of ectoine, respectively for strains D227 and D228 after the osmotic downshock process for 30 min in a solution containing 5% NaCl (Van-Thuoc *et al.* 2019). Interestingly, two bacteria in our study, i.e., K10 (5) and K10 (21) were able to release 85.5% and 91.9% of ectoine after the osmotic downshock using distilled water containing 1% w/v NaCl for 30 minutes. In addition, the bacteria isolated from the mangrove soil were also able to produce hydroxyectoine with an average ratio of hydroxyectoine to ectoine of around 1 to 5 (Van-Thuoc *et al.* 2019). Meanwhile, the average ratio of hydroxyectoine to ectoine produced by the halophilic bacteria in our study was higher by about 1 to 2.

Omara *et al.* (2019) reported ectoine production from halophiles obtained from various hypersaline ecosystems in Egypt. The selected halophiles obtained could produce ectoine with a yield of 10.88 to 28.62 mg/g cell dry weight. The most active ectoine-producers were identified as *Vibrio* sp. CS1 and *Salinivibrio costicola* SH3. The halophilic bacteria obtained in our study showed a higher yield of ectoine production of 10.1 to 105.4 mg/g cell dry

weight. Two of the best ectoine-producing bacteria in our study were also identified as *Salinivibrio costicola*, indicating that this bacterial species is one of the best ectoine producers. Interestingly, this study also proved the production of ectoine and hydroxyectoine from *Salinivibrio kushneri* that have not been reported to date.

Previous work reported ectoine production by *Halomonas elongata* BK-AG25 isolated from the mud crater of Bledug Kuwu, Central Java, Indonesia (Parwata *et al.* 2020). Using two-step cultivation, the bacteria produced 179.9 mg ectoine per g cell dry weight, higher than that produced by our halophilic bacteria in this study with the maximum yield of 105.4 mg/g cell dry weight. However, the best ectoine-producing halophilic bacteria obtained in this study, i.e. *Salinivibrio costicola* K10(3), *Salinivibrio costicola* K10(21), and *Salinivibrio kushneri* K10(5), have the potential to be optimized for the high-yield production of ectoine. A comparative genomic study has revealed that halophilic bacteria of genus *Salinivibrio* use ectoine to balance osmotic stress by de novo synthesis instead of transportation or accumulation from their environment (de la Haba *et al.* 2019). This gives the advantage that osmolyte content in the medium will not affect the synthesis of ectoine by bacteria.

Our study has found several halophilic bacteria with great potential to produce ectoine and hydroxyectoine. The bacteria were also able to excrete most of the ectoine and hydroxyectoine produced after the osmotic downshock process, hence very promising to be developed for industrial-scale production. The yield of ectoine and hydroxyectoine produced by the bacteria, however, should be further increased by optimizing the levels of nutrient and fermentation conditions.

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