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# Isolation and Characterization of New Ectoine-Producers from Various Hypersaline Ecosystems in Egypt

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## *ABSTRACT*

The biodiversity of aquatic and terrestrial ecosystems in Egypt including seas, lakes, and soils with high salinity levels provides rich habitats for flourishing halophilic microorganisms. The present study aims to isolate halophilic bacteria from sixteen samples representing ecosystems with various geographic locations and surveying the mechanisms by which they can tolerate the osmotic stress. Results revealed the highest salinity levels; sodium, chloride, sulfate, magnesium, calcium, and potassium concentrations in water sample of Wadi El-Natroun Lake since the total number of halophilic bacteria was 4.68/g and the largest number of halophilic genera was observed in the northern coast of Matrouh governorate. A total of 60 bacterial isolates selected during (June-October 2015) were screened using thin layer chromatography for osmolyte; ectoine. It has the ability to decrease the harmful effects of high salinity, heating, freezing, drying as well as oxygen radicals and other denaturing agents in halophiles. Quantitative estimation of ectoine by high-performance liquid chromatography showed its yield was ranged between 185 and 700 mg/l. Using biochemical tests and 16s rRNA technology, the most active ectoine-producers were identified as *Vibrio sp.* CS1 and *Salinivibrio costicola* SH3. The yielded ectoine was purified using cation exchange chromatography (Dowex 50 $WX_8$ resin) and its physicochemical properties were investigated using standard methodology. Characterization of purified ectoine via nuclear magnetic resonance and fourier transform infrared spectroscopy showed the similarity between bacterial ectoine and the authentic was nearly 99%. .

***KEYWORDS***

***Ectoine, Halophilic Bacteria, Hypersaline Environment, Osmotic Stress.***

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## INTRODUCTION

Although the diversity of natural microorganisms, a low fraction of the total microbial population has been isolated and characterized. Different environments with extreme conditions, such as high/low temperature, high/low pH, high salinity and high pressure, provide distinctive microorganisms with great potential for applications in food, chemicals, and drugs as well as environmental aspects **(Orellana *et al*., 2018).**

Halophiles (salt-loving) are referred to microorganisms that require salt for growing and can be found in hypersaline environments, which are widely distributed in various geographical areas on the earth, such as saline lakes, salt pans or salt marshes **(Setati, 2010)**. Halophilic microorganisms have been used in unlimited applications in different biological fields. Many halophiles can produce extracellular hydrolytic enzymes; amylases, lipases, proteases, xylanases, and cellulases which are termed as halophilic hydrolases and capable of catalyzing hydrolytic reactions under high salt concentrations and adverse conditions **(Rohban *et al*., 2009)**. Some halophiles synthesize polyhydroxyalkanoates (PHA); biodegradable polyesters that are industrially applicable in bioplastics, biofuels, and medicine **(Chen and Patel, 2012)**. The retinal proteins which participate in developing a variety of optical devices as photosensors are produced by halophilic archaea (haloarchaea) **(Grote *et al*., 2014)**. In addition, Halotolerant microorganisms play an important role in food fermentation for manufacturing food and food supplements **(Heldman *et al*., 2010)**. Successful bioremediation of oil spills has been observed in marine, Arctic, and Antarctic environments using heterotrophic, halophilic bacteria **(Rezaei *et al*., 2018)**. Osmolytes are intracellular compatible solutes that preserve the metabolism of halophiles under salt stress **(Burg and Ferraris, 2008)**. According to their chemical structure, they can be categorized into; polyols (glycerol, arabitol, mannitol, erythritol), sugars (sucrose, trehalose), betaines (trimethyl ammonium compounds), thetines (dimethylsulfonium compounds), amino acids (proline, glutamate, glutamine) and ectoines (ectoine, β-hydroxyectoine) **(Lentzen and Schwarz 2006)**. Ectoine is considered a natural cyclic tetrahydropyrimidine with low molecular weight and great water-binding capacity. Ectoine maintains the integrity of proteins, DNA and enzymes inside haophilic cells upon exposing to different stresses. Thus, it has extensive applications in enzyme technology and pharmaceutical industry **(Czech *et al*., 2018)**. In addition, ectoine is a very effective inhibitor for amyloid formation causing Alzheimer's disease and spongiform encephalopathies **(Ashraf *et al.*, 2014)**.

The objectives of this study are: (1) isolating new moderate halophiles from different hypersaline environments in Egypt; (2) investigating the type of compatible salts produced by isolated halophiles; (3) selecting the most-active ectoine producers; (4) characterizing the yielded ectoine by different chromatographic procedures.

## MATERIAL AND METHODS

### *Sampling*

Sixteen samples were collected from selected hypersaline environments including terrestrial and aquatic ecosystems; Abu kbeer, Ain Sokhna, Belbes, North coast, Port Said, Qaroun Lake and Wadi El-Natroun (*Zug-Hamra &* El-Beida) during (June-October, 2015). Soil samples were kept in sterile plastic bags whereas seawater samples were collected using 500ml sterile labeled bottles; they were transported under cooling and aseptic conditions, and stored at 4°C until analysis. The pH and temperature were determined immediately after sampling process whereas the salt content of selected samples was estimated within two days.

### *Isolation of halophilic bacteria*

Five milliliters of each water samples or five grams of each soil sample were placed in 50 ml sterile saline solution (0.9 %) and shaken for 1 h at 300 rpm. Ten-fold dilutions were made up to $10^{-7}$; 0.1 ml of each dilution was plated on Synthetic agar medium as previously described **(Hassan and Mahgoub, 2016)**. After incubation for 72h at 30°C, the number of growing halophiles was counted and expressed as Log of cell forming unit per gram. According to the morphological difference of growing halophiles; varied colonies were selected, purified and maintained for further studies.

### *Identification of selected halophiles (The most active ectoine producer)*

Two bacterial isolates were selected out of sixty that showing a large ectoine production with a little by-product and identified by biochemical characterization and 16s rRNA.

### *Biochemical characterization of halophilic isolates*

The two selected halophilic isolates were stained with Gram's stain and examined by light-microscope to characterize their morphology and facilitate their identification using biochemical tests; Catalase activity, Citrate utilization, Gelatin hydrolysis, Hydrogen sulphide test, Indole production, Methyl Red/ Voges-Proskauer Test, Motility test, Oxidase test and Urease test as well as sugar fermentation **(Azhar *et al.*, 2014)**.

The selected bacterial isolates were identified by sequence analysis of 16S RNA. Bacterial DNA was isolated from bacteria grown in Halophilic medium (HM) using the gene JET™ Genomic DNA Purification Kit (Fermentas, EU). PCR product was visualized by ethidium bromide using UV lamb transilluminator on 1% agarose gel and photographed using a digital camera (Sony). DNA amplification was carried out in 50 µl PCR mixture containing 25 µl Maxima Hot start PCR master mix (2x), 1 µM of each primer, 5 µl template DNA, 18µl water (nuclease free). PCR reaction was performed in Thermal cycler **(Thermo™)** using a pair of primer as following: forward: 5-AGA GTT TGA TCC TGG CTC AG-3, reverse 5-GGT TAC CTT GTT ACG ACT T-3 **(Wang and Wang, 1996)**. Each cycle started with an initial denaturation time (5 min) at 94°C followed by 35 cycles of amplification comprising a denaturation step for the 30s at 94°C, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min 30s. Reactions were completed with 10 min elongation at 72 °C. The PCR product was purified using the gene JET™ PCR purification kit. Sequencing of the amplified DNA was performed by Solgent Co. Ltd (Seoul, South Korea) using automated sequence analyzer ABI 3730xl. The sequence was checked for correction by chimera system using pintail program and compared to standard alignments available at NCBI database http://www.ncbi.nlm.nih.gov/BLAST. Then the phylogenetic tree was drawn using Mega-blast program version 6. The new nucleotide sequences were accessed in the GenBank under an accession number MK044835 and MK044836.

## ANALYTICAL PROCEDURES

### *Ectoine extraction*

Ectoine was extracted using two procedures according to purpose; solvent extraction (Ectoine quantification) and osmotic downshock (Ectoine purification). In the first procedure; growing cells were harvested by centrifugation (6000 rpm) for 10 min and washed twice with 50 mM Kpi buffer isotonic to the medium. Harvested cells were suspended in 80% (V/V) ethanol and allowed to stand for 12 h to extract the intracellular compatible solutes. The intracellular extract was filtered and evaporated at 50°C; the precipitate was re-suspended in a water/ chloroform (1:1v/v) mixture and mixed vigorously. The chloroform layer decanted and the upper clear layer was collected **(Zhu *et al.*, 2007)**. The second procedure is known as bacterial milking and based

on exposing bacterial cells to different salinity systems **(Sauer and Galinski, 1998)**. Due to osmotic shock, resting cells release ectoine to achieve osmotic equilibrium **(Teixidó *et al*., 2005)**. This step is repeated to achieve maximum ectoine extraction before removing distorted cells and collecting the supernatant containing ectoine.

### *Ectoine quantification*

Ectoine was determined by a high-performance liquid chromatography located at the Regional Center for Mycology and Biotechnology (Al-Azhar University, Cairo), using a C18 column and a mobile phase composed of acetonitrile and water (8:2) with 1ml/min flow rate. Ectoine was detected at 210 nm and the concentration was calculated by the calibration curve constructed from the known concentrations of standard ectoine **(Eshinimaev *et al*., 2007)**. In addition, ectoine was estimated indirectly by determining its hydrolysis products; N-alpha-Acetyl-diaminobutyric acid (DABA) using a colorimetric reaction with ninhydrin. The crude ectoine dissolving in 1 ml KOH (0.1 M) is hydrolyzed for 20h at 50°C; the hydrolysis product reacts with 100 µl ninhydrin (1% in absolute ethanol). Thereafter, this mixture was heated at 100°C in a water bath for 15 min, cooled at room temperature and its absorbance was estimated at 570 nm **(Ono *et al*, 1998)**.

Concentration of ectoine (mg/ml) = $O.D_{570}/0.054$

### *Ectoine purification and characterization*

Protein decontaminated residues are removed from the crude ectoine solution by decreasing the pH to 1.4. The aqueous ectoine solution was purified using a Dowex 50 $WX_8$ cation exchange resin ($Na^+$ form) packed in a 1.4×9 cm column. Ectoine was bound to the cation exchange resin where impurities were removed by washing with HCl (1.4 M). Subsequently, ectoine was eluted with 0.1 M NaOH and collected fractions were monitoring by UV-spectrophotometer until neutralization of eluted solution; the absorbance intensity of eluted fractions at 230 nm increased with increasing ectoine concentration. The partially purified fractions containing ectoine was lyophilized using a Modulyo® lyophilizer (Edwards, Sussex, England) and kept at -4°C **(Sauer and Galinski, 1998)**.

Ectoine dissolved in methanol was scanned by UV-Visible spectrophotometer in the range between 200 nm to 800 nm (**Eshinimaev *et al*., 2007)**. The partially purified fractions containing ectoine was concentrated and loaded on glass plates coated with F60 silica gel that previously activated by heating for 105ºC for 1h **(Vargas *et al*., 2006)**. Then, fractions were developed in a solvent system composed of n-butanol, water, and acetic acid in the ratio of (12: 5: 3 V/V/V). The purified ectoine was detected by spraying the developing spots by 0.2% ninhydrin dissolved in ethanol and heating for 5 min at 130°C. Finally, $R_f$ value of purified ectoine was determined in the reference of authentic ectoine purchased from Sigma Company **(Ono *et al*., 1998)**.

In addition, ectoine was characterized by $^1H$ NMR, Mercury-300BB located at Micro Analytical Center, Faculty of Science, Cairo University using deuterated chloroform at 300 MHz. FT-IR analysis of ectoine was performed at Infrared lab, NCRRT, Egyptian atomic energy authority (A Bruker model Tensor 27 spectrometer, Bruker Corp., Billerica, MA) between 500 and 35000 ($cm^{-1}$).

### *Determination of other osmolytes*

The halophilic isolates were streaking on HM **(Quillaguamán *et al*., 2004)** and incubated at 30°C for 24h Then, the production of polyhydroxyalkonates was estimated by 0.02% Sudan Black B in ethanol; the dark blue colour of colonies is a positive result **(Gudmalwar and Kamble, 2014)**. In addition, the total carbohydrates derivatives and free amino acids contents were determined using the phenol-sulfuric acid method and Kjeldahl procedures; respectively **(Dawson *et al*., 1986 and Reddy *et al*., 1983)**.

## RESULTS

### *Physicochemical characteristics of various samples*

In the present study, sixteen samples were collected from hypersaline environments found in Egypt including terrestrial and aquatic ecosystems (Table 1). Water samples were collected from the Mediterranean Sea, Red Sea, Suez Canal and hypersaline lakes. In addition, five soil samples out of eight were selected from seawater sediments (beneath the water surface by 50cm) and two samples were collected from areas weren't previously planted due to high salinity content but they are nearby cultivated agricultural fields whereas only one sample represents saline mud. The data summarized in table (1) showed the physical characteristics of hypersaline environmental samples from different geographical regions in Egypt (Alexandria, El-Beheira, El-Sharkia, Matrouh, El-Fayoum, Port Said, El-Suez and South Sinai). Temperature and pH values of these samples are in a range of 31-39°C and 6.1-8.8; respectively.

**Table (1) :** *Physical characteristics of hypersaline environmental samples*.

| Sample code | | Geographical location | Sample type | Physical characteristics | | |
|---|---|---|---|---|---|---|
| | | | | Color | pH | Temp (°C) |
| Terrestrial ecosystems | BS | Belbes- El-Sharkia | Clay soil | Brownish Black | 6.1 | 35 |
| | CA | North coast- Matrouh | Loamy soil | Brownish Black | 6.5 | 39 |
| | CS | North coast- Matrouh | Sandy soil | Bright Creamy | 6.2 | 37 |
| | KS | Abu kbeer- Ei-Sharkia | Sandy soil | Dark Yellow | 6.2 | 38 |
| | PS | Port said- Port said | Saline mud | Dark Black | 6.3 | 30 |
| | QS | Qaroun Lake- El-Fayoum | Sandy soil | Bright Golden | 6.3 | 38 |
| | RS | Wadi El-Natroun- El-Beheira | Sandy soil | Brownish Black | 7.9 | 39 |
| | S | Ain Sokhna-El-Suez | Sandy soil | Yellowish | 7.0 | 37 |
| Aquatic ecosystems | A | Alexandria | Seawater | Colorless | 6.3 | 31 |
| | BW | Belbes- El-Sharkia | Well-water | Colorless | 6.3 | 35 |
| | CW | North coast- Matrouh | Seawater | Colorless | 6.5 | 39 |
| | KW | Abu kbeer- El-Sharkia | Seawater | Colorless | 6.3 | 38 |
| | QW | Qaroun Lake- El-Fayoum | Seawater | Yellowish | 6.2 | 38 |
| | RW | Wadi El-Natroun- El-Beheira | Lake water | Colorless | 8.8 | 39 |
| | SH | Sharm El-Sheikh-South Sinai | Lake water | Colorless | 6.7 | 38 |
| | W | Wadi El-Natroun- El-Beheira | Lake water | Colorless | 8.7 | 39 |

Table (2) shows that the highest salinity contents of soil samples are observed in RS sample which was collected from sediments of Wadi El-Natroun lake (Zug-Hammra; Image 1a) whereas the lowest ones are estimated in BS sample. Analysis of PS sample (mud saline soil) reveals the highest value of calcium, magnesium, chloride, sulfate, sodium, and potassium ions. Similar to soil samples, the highest salinity content; 17.6% was estimated in water sample "W" collected from Wadi El-Natroun (El-Beida; Image 1b) whereas the highest levels of chloride, carbonate, bicarbonate, sulfate, and sodium are measured in RW sample that also collected from Wadi El-Natroun lakes (Table 2). These concentrations are also higher than their comparable values in soil samples. The highest values of calcium and magnesium are estimated in samples A and KW; respectively whereas the lowest values are found in RW and SH samples, on the same order.

**Table (2) :** *Chemical characteristics of soil sediment samples.*

| Sample code | | Salinity (%) | Salt concentration (PPM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $Ca^{+2}$ | $Mg^{+2}$ | $Cl^{-1}$ | $CO^{-2}_3$ | $HCO^{-}_3$ | $SO^{-2}_4$ | $Na^{+1}$ | $K^{+1}$ |
| *Terrestrial* | BS | 1.4 | 17.8 | 11.0 | 41.0 | N.D* | 6.0 | 30.9 | 58.0 | 0.7 |
| | CA | 1.8 | 3.3 | 3.0 | 82.5 | N.D | 8.0 | 1.2 | 80.0 | 0.9 |
| | CS | 3.1 | 2.6 | 4.5 | 80.0 | N.D | 5.0 | 0.9 | 82.0 | 1.1 |
| | KS | 2.9 | 2.2 | 6.5 | 92.0 | N.D | 5.5 | 7.1 | 110.0 | 1.3 |
| | PS | 13.2 | 24.7 | 11.3 | 425.0 | N.D | 5.0 | 121.5 | 225.0 | 2.5 |
| | QS | 3.6 | 2.8 | 4.9 | 111.0 | N.D | 6.5 | 0.51 | 122.0 | 1.4 |
| | RS | 6.4 | 3.2 | 3.8 | 151.0 | 3.0 | 12.5 | 0.24 | 132.0 | 1.5 |
| | S | 2.3 | 2.9 | 6.4 | 128.0 | N.D | 8.5 | 0.14 | 111.0 | 1.3 |
| *Aquatic* | A | 5.2 | 17.6 | 11.2 | 125.0 | 3.5 | 71.3 | 11.2 | 120.0 | 1.5 |
| | BW | 0.13 | 3.6 | 3.9 | 8.0 | 0.6 | 0.02 | 3.9 | 6.1 | 0.2 |
| | CW | 5.9 | 17.4 | 0.43 | 127.0 | N.D | 73.5 | 0.43 | 110.0 | 1.4 |
| | KW | 6.1 | 16.9 | 12.9 | 135.0 | N.D | 68.7 | 12.9 | 130.0 | 1.9 |
| | QW | 3.7 | 15.9 | 7.9 | 98.0 | N.D | 207.7 | 7.9 | 100.0 | 1.7 |
| | RW | 16.1 | 0.05 | 3.0 | 276.0 | 330.0 | 708.7 | 3.0 | 150.0 | 2.9 |
| | SH | 4.8 | 16.7 | 0.35 | 144.0 | 4.2 | 72.5 | 0.35 | 128.0 | 1.7 |
| | W | 17.6 | 1.3 | 2.5 | 269.0 | 240.0 | 711.0 | 2.5 | 146.0 | 2.5 |

**N.D: Not Detected*


**Image 1:** Photo of hypersaline environmental samples

A: Zug-Hammra, Wadi El-Natroun B: El-Beida, Wadi El-Natroun

### *Isolation of moderately halophilic bacteria from terrestrial and aquatic ecosystems*

Isolation of halophilic bacteria from their natural habitats requires adaptation process before they are able to grow on synthetic media with high salt concentration (10%). Table (3) shows the viability of moderately halophilic bacteria in selected samples on synthetic medium at 30°C. RS sample that was collected from soil salt marsh from Wadi El-Natroun shows the highest total bacterial count; $6.4\times10^4$ CFU/g (Log N: 4.81) whereas the lowest total bacteria count (Log N: 1.79) was isolated from PS sample that has the highest chloride concentration as shown in table (2). Thirty-four bacterial isolates were selected from soil samples according to their morphological difference, purified and preserved on LB me-

dium at 4°C. These bacterial isolates were symbolized referring to their origin (soil samples) as shown in table (3). Regarding aquatic ecosystem, twenty-six bacterial isolates were also selected purified and preserved on Luria-Bertani medium **(Hassan and Mahgoub, 2016)** at 4°C. The maximum halophiles count was observed in RW sample; $4.8\times10^4$ (CFU/ml) that are collected from Zug-Hammra (Wadi El-Natroun Lake) whereas the lowest halophile count was showed in "A sample" ($2.2\times10^2$ CFU/ml).

**Table (3) :** *Isolation of moderately halophilic bacteria from terrestrial and aquatic ecosystems*.

| Sample code | | Total bacterial count | | No of isolated halophiles* | Symbol of selected halophiles |
|---|---|---|---|---|---|
| | | CFU/g | Log N | | |
| *Terrestrial* | BS | $4.4X10^2$ | 2.64 | 1 | $BS_1$ |
| | CA | $5.0X10^4$ | 3.70 | 10 | $CA_1$, $CA_2$, $CA_3$, $CA_4$, $CA_5$, $CA_6$, $CA_7$, $CA_8$, $CA_9$, $CA_{10}$ |
| | CS | $4.2X10^3$ | 3.62 | 6 | $CS_1$, $CS_2$, $CS_3$, $CS_4$, $CS_5$, $CS_6$ |
| | KS | $2.1X10^4$ | 4.32 | 2 | $KS_1$, $KS_2$ |
| | PS | 6.2x10 | 1.79 | 3 | $PS_1$, $PS_2$, $PS_3$ |
| | QS | $5.3x10^2$ | 2.72 | 2 | $QS_1$, $QS_2$ |
| | RS | $6.4x10^4$ | 4.81 | 6 | $RS_1$, $RS_2$, $RS_3$, $RS_4$, $RS_5$, $RS_6$ |
| | S | $3.5x10^3$ | 3.54 | 4 | $S_1$, $S_2$, $S_3$, $S_4$ |
| *Aquatic* | A | $2.2X10^2$ | 2.34 | 2 | $A_1$, $A_2$ |
| | BW | $4.5X10^2$ | 2.65 | 2 | $BW_1$, $BW_2$ |
| | CW | $2.5X10^4$ | 4.40 | 4 | $CW_1$, $CW_2$, $CW_3$, $CW_4$ |
| | KW | $1.5x10^4$ | 4.18 | 2 | $KW_1$, $KW_2$ |
| | QW | $4.3x10^3$ | 3.63 | 2 | $QW_1$, $QW_2$ |
| | RW | $4.8x10^4$ | 4.68 | 6 | $RW_1$, $RW_2$, $RW_3$, $RW_4$, $RW_5$, $Rw_6$ |
| | SH | $2.5x10^4$ | 4.40 | 4 | $SH_1$, $SH_2$, $SH_3$, $SH_4$ |
| | W | $4.0x10^4$ | 4.60 | 4 | $W_1$, $W_2$, $W_3$, $W_4$ |

** According to morphological characteristics*

### *Screening the most potent selected isolates for ectoine production*

A total of 60 halophilic isolates (selected during June-October 2015) were screened for ectoine production. In a preliminary experiment, the intracellular extracts of these isolates after growing on HM medium supplemented with 10 % NaCl at 30°C for 72 h under shaking conditions (150 rpm) were analyzed chromatographically using a thin layer. Ectoine-production is cleared as violet-red spots with 0.3 $R_f$ value; this $R_f$ is comparable to standard ectoine (plate 1). Results revealed 15 bacterial isolates out of 60 could produce ectoine as compatible solute. HPLC studies (Table 4) revealed the superiority of a moderate halophilic isolate (CS1) that selected from saline soil of the North Coast of Egypt (Matrouh governorate) in ectoine production (28.62 mg/g) followed by SH3 which isolated from a water sample of Red sea (27.83 mg/g) whereas the lowest ectoine yields were estimated by BW2, CA1 and CA9 halophiles (less than 15 mg/g).

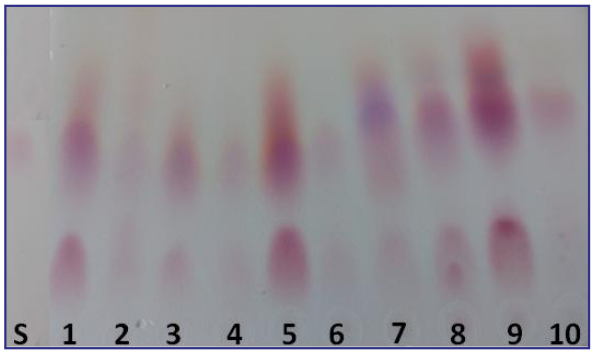


**Plate 1:** TLC plate showing ectoine as violet-red spots with Rf value 0.3
S: Standard ectoine
1-10: Intracellular extracts of selected halophiles

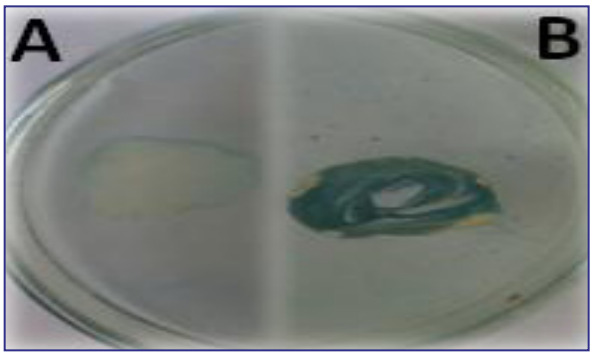


**Plate 2:** Estimation of PHA production using Sudan black stain
A: -ve PHA production
B: +ve PHA production

**Table (4) :** *Screening of selected halophilic bacteria for compatible salts production.*

| Symbol of Selected halophiles | Ectoine yield (mg/g) | Other compatible salts | | |
|---|---|---|---|---|
| | | Polyhydroxy Alkonate (PHA) | Carbohydrate derivatives (mg/g) | Free amino acids derivatives (mg/g) |
| **BW1** | 17.10 | - | 1.22 | 3.28 |
| **BW2** | 10.88 | - | 5.00 | 1.94 |
| **CA1** | 11.92 | - | 3.48 | 27.06 |
| **CA2** | 20.89 | + | 3.42 | 17.63 |
| **CA5** | 22.35 | - | 3.82 | 4.75 |
| **CA9** | 13.33 | + | 1.93 | 5.04 |
| **CS1** | 28.62 | - | 4.25 | 1.10 |
| **CS2** | 17.85 | + | 1.70 | 9.55 |
| **CS5** | 19.64 | + | 4.30 | 39.06 |
| **CS6** | 23.92 | - | 3.27 | 2.52 |
| **CW1** | 15.74 | + | 5.44 | 71.63 |
| **KW2** | 24.00 | - | 12.31 | 64.20 |
| **QW2** | 22.93 | - | 3.39 | 15.22 |
| **S2** | 17.27 | + | 5.14 | 20.59 |
| **SH3** | 27.82 | - | 3.72 | 6.28 |

To choose the best candidate for ectoine production in the industrial point of view, the present study assumed a strategy based on selecting ectoine with minimum amounts of by-products or without by-products at all. It's known that moderate halophilic bacteria can accumulate sugars, Polyhydroxy Alkonate (PHA) and free amino acids under stress conditions as compatible solutes. The selected halophiles (15 bacterial isolates) were tested for PHA production; results showed six bacterial isolates (CA2, CA9, CS2, CS5, CW1, and S2) were able to produce PHA (Table 4). Plate (2) showed the positive result of PHA production as estimated by Sudan black stain. In addition, the yield of osmolytes with sugar-origin is ranged between 1.22 mg/g (BW1) and 12.31 mg/g (KW2) as shown in table (4). The

contamination of ectoine with amino acids derivatives is another technical problem facing the industrial production of ectoine by halophiles. Table (4) also shows the free amino acid content is ranged between 1.10 mg/g (CS1) and 71.63 mg/g (CW1).

### *Identification of the most active ectoine producers*

#### Molecular identification of the most active ectoine producers

The polymerase chain reaction (PCR) is a powerful tool for identifying bacteria and commonly used to amplify 16S rRNA genes **(Yeung *et al.*, 2009)**. According to the results, the 16S rRNA gene of the most active ectoine producers CS1 and SH3 were amplified successfully with 1.5 kb band size (Image 2). The PCR products were sent to Solgent Co. Ltd (Seoul, South Korea) for purification and sequencing using automated sequence analyzer.

The result sequences 1166 and 1051 bp for selected halophiles CS1 and SH3; respectively were assembled with genomes available at NCBI database (www.ncbi.nlm.nih.gov).

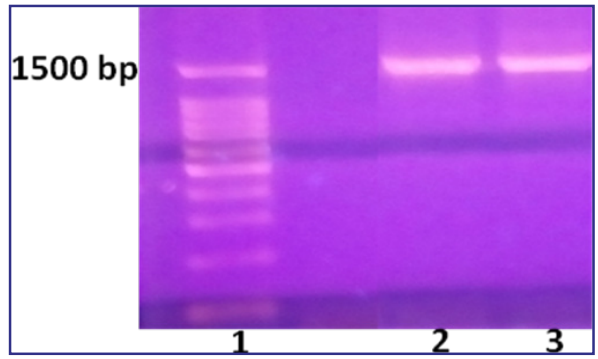


**Image (2):** Agarose gel electrophoresis for PCR products of amplified 16S rRNA (approximately 1.5 kbp)
Lane (1): DNA molecular weight marker (1 kb)
Lane (2): Amplified PCR products of Vibrio sp. CS1
Lane (3): Amplified PCR products of Salinivibrio costicola SH3

The 16S rRNA sequencing proved the high similarity of selected halophiles CS1 (97%) with *Vibrio sp*. Q09 (accession number KY818842.1) and of SH3 (99%) with *Salinivibrio costicola* pb-WC11147 (accession number JX913853.1). Figure (1) clears the phylogenetic trees of selected halophiles using blast program; the new sequences were registered at NCBI with accession numbers MK044835 and MK044836 on the same order.

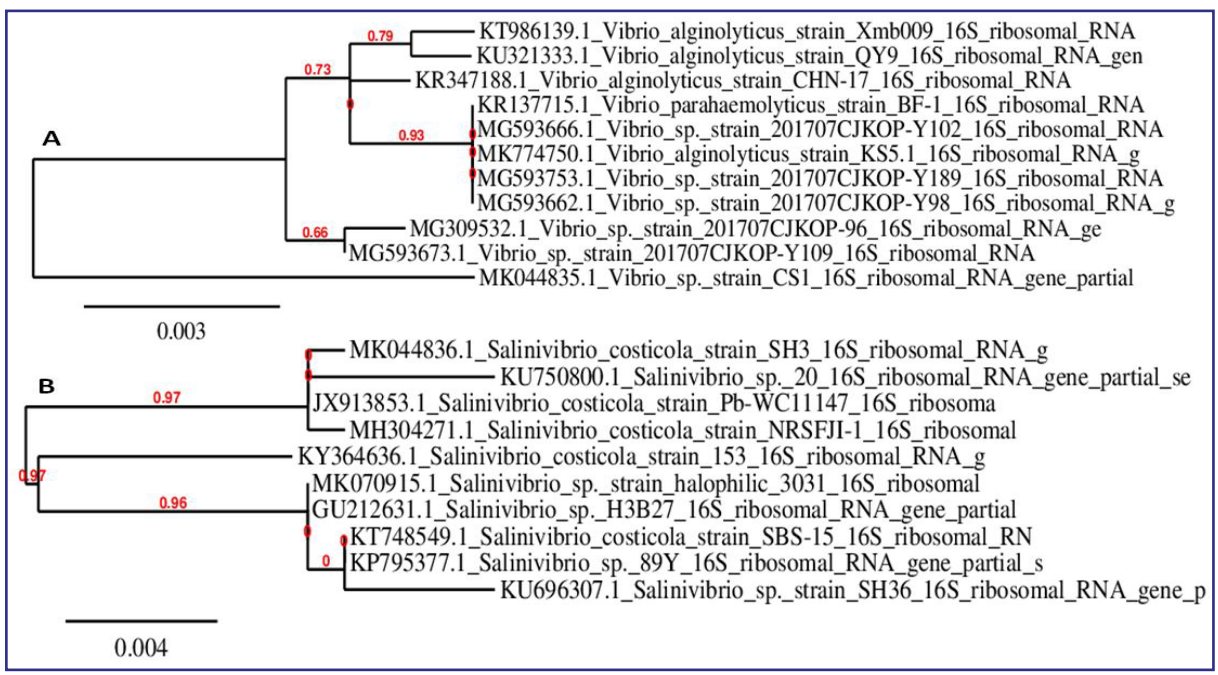


**Figure (1):** Neighbour-joining phylogenetic trees of selected halophiles
(A) Vibrio sp. CS1 (B) Salinivibrio costicola SH3.

***Physiological characterization and biochemical features most active ectoine producers***

Phenotype methods are classical methods used in laboratories for classification and identification of microorganisms based on their morphological and biochemical characterization **(Donelli *et al*., 2013)**. Colonies of CS1 (*Vibrio sp*.) are flat, circular with smooth margins and has no reverse pigmentation. It is gram-negative, non-motile short rod with moderate salinity tolerant (15%) but it can't grow in the absence of NaCl. It has the ability to grow in a wide range of temperature (15-40°C) and pH values (5.5-10.0). It also shows positive results with catalase, gelatin liquefication, methyl red, and citrate utilization and negative results with oxidase, urease, indole and hydrogen sulfide production and Vogas-Proskaeur (Table 5).

Regarding SH3 (*Salinivibrio costicola*); it is convex, circular and creamy in color as well as motile, rod-shaped and gram-negative bacteria. It able to grow under a wide range of NaCl (0.5 to 20%) and can't grow in the absence of NaCl. It has the ability to grow in a wide range of temperature degrees (15-50°C) and it grows better under alkaline conditions (5.5-10.0). It is positive for catalase and gelatin liquefaction and negative for oxidase test, urea, and $H_2S$ production. IMVIC test showed that it is positive for indole production, Vogas-Proskaeur and citrate utilization while negative for methyl-red (Table 5).

**Table (5) :** *Screening of selected halophilic bacteria for compatible salts production***.**

| Characteristic features | *Vibrio sp.*CS1 | *S. costicola* SH3 |
|---|---|---|
| **Gram-Stain** | Negative | Negative |
| **Cell morphology** | Short rod | Short rod |
| **Cell pigmentation** | Creamy | Creamy |
| **Motility** | Non- motile | Motile |
| **NaCl tolerance (%)** | 0.5 - 15 | 0.5 – 20 |
| **pH tolerance** | 5.5 – 10.0 | 5.5 – 10.0 |
| **Temperature tolerance (°C)** | 15 – 40 | 15 – 50 |
| **Catalase test** | + | + |
| **Citrate utilization** | + | + |
| **Gelatin hydrolysis** | + | + |
| **$H_2S$ production** | - | - |
| **Indole production** | - | + |
| **Methyl red test** | + | - |
| **Oxidase test** | - | - |
| **Voges-Proskaeur test** | - | + |
| **Urease test** | - | - |
| **Sugar fermentation** | | |
| Galactose | + | - |
| Glucose | + | + |
| Lactose | - | - |
| Mannitol | + | + |
| Mannose | + | + |
| Melibiose | - | - |
| Sucrose | + | + |

In addition, the fermentation abilities of selected halophiles were examined for different sugar substrates (galactose, glucose, lactose, mannitol, mannose, melibiose and sucrose). Table (5) shows the positive fermentation ability (acid and gas production) of *Vibrio sp.* CS1 for galactose, glucose, mannitol, mannose, and sucrose and negative fermentation ability for lactose and melibiose. Regarding *Salinivibrio costicola* SH3, it shows fermentation ability for glucose, mannitol, mannose, and sucrose only.

### *Purification of ectoine*

The acidified concentrated crude ectoine was eluted with HCl solution (pH 1.4) to remove the uncharged and negatively charged compounds. Afterward, purified ectoine was eluted with 0.1 M NaOH. Twelve fractions were collected from the elution and the collection process was stopped when the pH of the solution reached neutral. Ectoine fractions were monitored by UV-Visible spectroscopy at (230 nm). Screening process revealed the absence of ectoine from the first nine fractions and the other fractions that showed a clear absorbance at 230 nm were stored for ectoine characterization.

### *Characterization of ectoine*

Physical description of purified ectoine (after cation exchange chromatography) is completely different from the crude one that released from cells of selected halophiles (*Vibrio sp.* CS1 and *Salinivibrio costicola* SH3). Crude ectoine is a massive cake containing large percent of NaCl whereas purified one has a colorless fine needle crystal shape. The solubility of ectoine in different organic solvents; acetone, chloroform, absolute ethanol, isopropyl alcohol, methanol, n-heptane, toluene as well as water were examined. Results revealed the high solubility of ectoine in water, followed by methanol and finally ethanol and isopropyl alcohol. In addition, it is insoluble in acetone, chloroform, n-heptane, and toluene. Thus, organic solvents as ethanol and methanol cannot be used individually for extracting ectoine but the mixture of water and ethanol or methanol is more effective.

The absorbance spectrum of purified ectoine was examined using UV-Visible spectrophotometer (200- 1000 nm). The spectrum of ectoine dissolved in methanol showed that there is only one strong peak observed at the ultra-violet region and the absorption spectrum starts at 195 nm and ends at 230 nm with maximum intensity at 208 nm (Fig. 2). $^{1}$H NMR is a rapid method for analyzing and identification of ectoine. In the present study, purified ectoine was analyzed by $^{1}$H NMR using $D_2O$ as a mobile solvent. $^{1}$H-NMR spectrum shows the hydrogen atoms peaks of purified ectoine is the same as the analogue of authentic one (purity > 99%) at 2–5 ppm (Figure 3). The purified ectoine and authentic ectoine were scanned using Fourier transform infrared spectroscopy (FTIR), between 500 and 35000 ($cm^{-1}$). Figure (4) shows a similar fingerprint between the bacterial ectoine and authentic one.

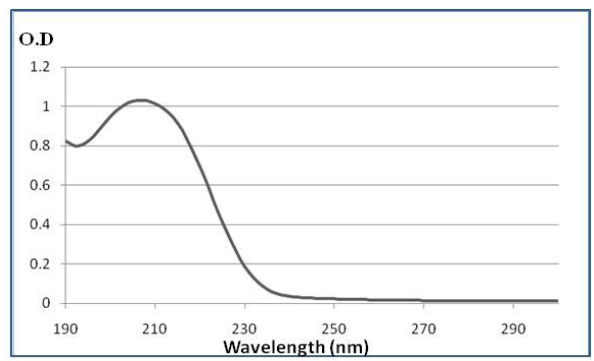


**Figure (2):** UV-spectrum of ectoine dissolved in methanol.

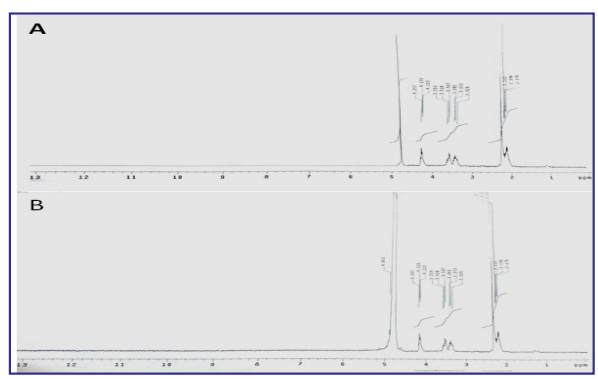


**Figure (3):** $^{1}$H -NMR spectrums
A: Authentic Ectoine and B: Purified Ectoine.

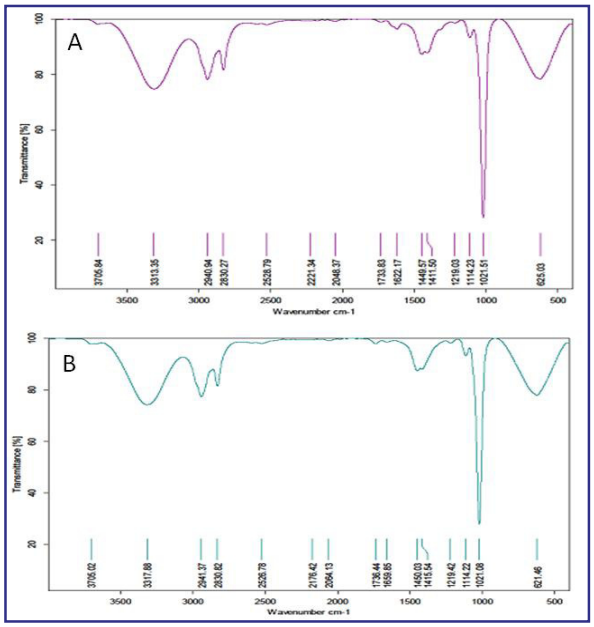


**Figure (4):** FTIR spectrums
A: Authentic Ectoine B: Purified Ectoine.

## DISCUSSION

Hypersaline environments with high salinity comprising soil and water are widespread all over the world. Oceans are the largest hypersaline environments constituting approximately 72% of the biosphere and contain more than 3.5% total dissolved salts. The salinity system of seawater depends on the concentrations of dissolved salts including $Na^{+}$, $Cl^{-}$, $SO_4^{-2}$, $Mg^{+2}$, $Ca^{+2,}$ and $K^{+}$ that varied by a balance between evaporation and precipitation **(Nessim *et al.*, 2015)**. In addition, the salinity of hypersaline lakes is seasonally varied; it is increased in summer by evaporation and decreased in winter due to rainfalls. The salinity system in Wadi El-Natroun is distinctive to the other salinity ecosystems prevalent in Egypt especially their physical properties and chemical composition. **Taher (1999)** determined the salinity of Wadi El-Natroun lakes is ranged from 283 to 540 g/l. In addition, Hydrogen ion concentration (pH-value) of Wadi El-Natroun samples is alkaline due to the presence of a high concentration of carbonate whereas pH values of various salinity ecosystems in Egypt are almost neutral **(Abdelmongy and El-Moselhy, 2015)**.

The present study succeeds to isolate 60 halophilic bacteria from biodiversity ecosystems in Egypt either terrestrial or aquatic environment. On the same trend, **Osman *et al.*, (2009)** isolated halophilic bacteria *Halomonas sp.* EG6 from Burg El-Arab Lake, Egypt. That strain was tolerant to high NaCl concentration up to 4 M and able to synthesis ectoine as the main compatible solute with moderate productivity; 3.7 g/l after 7 days of osmotic downshock. **Elbanna *et al.*, (2015)** selected *Halobacterium sp.* HP25 out of 33 halophilic bacterial isolates from Qarun Lake, Egypt. Its growth yield, as well as protease production, was maximized at 30% NaCl (W/V). **Nada *et al.*, (2011)** isolated one-hundred bacteria from different saline soils of Wadi El-Natroun, the North coast, Giza and El-Sharqia governorates. These isolates belonged to *Halococcus salifodinae*, *Pseudomonas aeruginosa*, *Pseudomonas frourescens*, and *Pseudomonas mallei*. Out of them, four isolates are salt-tolerance due to the expression of EctC gene that responsible for ectoine production. Alternatively, thalassohaline hypersaline environments; saltern pond as Wadi El-Natroun lake may include communities survive salinity up to 30% with dominance to *Acinetobacter, Alcaligenes, Alteromonas, Flavobacterium, Pseudomonas*, and *Salinivibrio* **(Ventosa *et al.*, 1998)**

Ectoine is one of the compatible solutes that synthesized by different types of bacteria in hypertonic environments to overcome osmotic stress **(Gao *et al.*, 2014)**. The present study aims to select local halophilic isolates having ectoine as the predominant compatible solute. Thin layer chromatography shows 25% of selected halophiles synthesize ectoine with $R_f$ equals 0.3. Similarly, **Vaidya *et al.*, (2018)** identified ectoine in halophilic bacterial strains; *Halobacillus trueperi* SS1 and *Halobacillus trueperi* SS3 using TLC as orange-red spots with 0.32 $R_f$ value. Selected ectoine producers are belonging to Vibrio and Salinivibrio genera, the latter genus is constructed for differentiating between *Salinivibrio costicola* and Vibrio species. *Salinivibrio costicola*

is facultative anaerobic Gram-negative bacteria and haloalkaliphilic as well as motile and non-spore-former. *Salinivibrio costicola BAG* strain isolated from a saline lake in Ras Mohammed (Egypt) grew optimally at 35°C with salinity tolerance up to 16% and it is tolerant to pH values between 6.0 and 10.0 **(Romano *et al*., 2011)**.

The accumulation of different osmolytes inside bacterial cells depends on the type of species and growth conditions **(Joghee and Jayaraman, 2016)**. **Mothes *et al*., (2008)** found that *Halomonas elongata* simultaneously synthesized PHA and ectoine under stress conditions during shortage of nutrients and high osmotic pressure. After three days of incubation, their yields were 50% and 14%; respectively at 10% NaCl. **Van-Thuoc *et al*. (2010)** studied the co-production of ectoine and biopolyester poly (3-hydroxybutyrate) (PHB) by *Halomonas boliviensis* in two fed-batch cultures. In the first stage, high cell mass was achieved whereas high yields of ectoine and PHB were maximized in the second stage. At 7.5% NaCl, PHB and ectoine contents represented 68.5% and 7.2%. With higher NaCl concentration (12.5%), the ectoine content increased up to 17%. However, the yield of ectoine in this co-production process isn't cost-effectiveness **(Chen *et al*., 2014)**. Alternatively, in the present study, the isolated halophiles having the ability to produce PHA are excluded during the selection process. Carbohydrate, sugars and their derivatives as trehalose, sucrose, glycerol, and arabitol play a role as compatible solutes in halotolerant bacteria especially trehalose that used as a cryoprotectant for freeze-drying of bio-molecules **(Margesin and Schinner, 2001)**. It's found that *Halorhodospira halochloris* accumulated three compatible solutes; glycine betaine, trehalose, and ectoine; the latter solutes were synthesized under carbon limitation while the former one under nitrogen shortage **(Roberts, 2005)**.

Selecting candidates synthesize ectoine with low amino acid derivatives is the final step in screening process. **Oren (2006)** found that *Halomonas elongata* produce $K^+$ glutamate as the major compatible solute at 3% NaCl or less whereas, at higher salinities, ectoine is the predominant compatible solute. On the same trend, it is mentioned that *Virgibacillus pantothenticus;* the Gram-positive bacteria requires pantothenic acid for growth; synthesized the compatible solutes proline and ectoine under salt stress. The moderate salinity stress was accompanied by rising in proline concentration whereas ectoine was dominated at higher regimes. This phenomenon has termed as "osmolyte switching" **(Kuhlmann *et al*., 2011)**.

In the present study, purification of ectoine was based on cation exchange chromatography using appreciate resin (negatively charged polymer) in which act as a medium for positive ion exchange **(Shivanand and Mugeraya, 2011)**. Crude ectoine was acidified with concentrated HCl to convert zwitterionic ectoine (pH 7) to cationic form (pH 1.4) as well as to remove protein residues and solid impurities **(Rui-Feng *et al*., 2017)**. **Onraedt *et al*., (2005)** mentioned that the purification process of ectoine depends on the difference between the solubility of NaCl and ectoine in 80% ethanol since NaCl exists with crude ectoine as a contaminant. NMR spectroscopy is a powerful technique for identifying the chemical molecular structure of various natural-origin compounds in which the atomic nuclei of any biomolecule can be oriented in the presence of a strong magnetic field with characteristic frequencies **(Motta *et al*., 2004)**. According to previous reviews, peaks at 2.17 – 2.20 ppm arise from $H_3$ and $H_6$; respectively whereas peaks at 3.3 – 3.5 ppm and 4.1 ppm represent $H_4$ and $H_2$ on the same order. Methyl group linked to pyrimidine range frequently appears at 2.5 ppm (**Kondepudi and Chandra, 2011**). **Ongagna-Yhombi and Boyd (2013)** also examined the production of ectoine and glutamate as osmolytes by *Vibrio parahaemolyticus* in response to different salinity stress (1, 3 and 6% NaCl) using $^1$H NMR.

No peaks for compatible solutes were detected in the spectrum at 1% NaCl where proton peaks of other metabolic products are predominant. On the other hand, peaks of glutamate and ectoine were detected at 3 and 6% NaCl, and the intensity and size of ectoine peak are increased over glutamate as increasing NaCl concentrations. Alternatively, **Tanne *et al*., (2014)** differentiate between ectoine and hydroxyectoine using FTIR spectrometer. Both spectra contain broad overlapping bands in the hydrogen stretching region N–H, C–H and N–H (>2500cm$^{-1}$). The fingerprint of hydroxyectoine clears its richness by OH that attached to heterocyclic ring thus the bands at 1088 cm$^{-1}$ does not exist in the ectoine spectrum.

In conclusion, isolating industrial candidates from new environmental resources acts as a potent strategy for selecting microbial strains with higher metabolites yield. Thus, the present study succeeds to select bacterial strains with potent ectoine productivity and a lower content of other osmolytes whatever their nature, polyhyhroxy alkonate free amino acids or polysaccharides.

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