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Egypt. J. Bot. **Vol. 59,** No. 2, pp. 537 - 549 (2019)

Adaptive Changes in Saturated Fatty Acids as a Resistant Mechanism in Salt Stress in *Halomonas alkaliphila* YHSA35

Yasser El-Halmouch

Department of Botany and Microbiology, Faculty of Science, Damanhour University, Damanhour, Egypt.

SINCE salinity is one of the major abiotic stresses, bacteria totally have different adaptive or tolerant mechanisms that respond to salinity stress. Multiwavelength UV-Vis spectroscopy used to estimate the bacterial growth under different sodium chloride concentrations. Absorbance ratio of 280nm to 260nm (A_{280}/A_{260}) varied considerably according to the salt concentration. This showed some metabolic activity changes as a part of the adaptive response that allows *Halomonas alkaliphila* to face salinity stress changes. Here, adaptive changes of fatty-acid composition of *H. alkaliphila* YHSA35 because of different sodium chloride concentrations were determined. In this work, fatty acids methyl esters (FAME) composition analysis was achieved and estimated the presence of thirty-four fatty acids in *H. alikaliphila* YHSA35 cells. Quantitatively changes were found within the level of saturated fatty acids; Caproic, Lauric, Undecanoic, Myristic, Palmitic, Heptadecanoic and in unsaturated fatty acids; Oleic, cis-11-Eicosenoic, Erucic. In high salt concentration, unsaturated fatty acids synthesis rate is reduced, resulting in an accumulation of palmitic acid. In conclusion, levels of saturated fatty acid profile changed in *H. alkaliphila* YHSA35 because of salinity stress that may modulate the membrane lipid viciousness for adaptation and best cellular perform.

Keywords: Salt stress, Salinity, *Halomonas alkaliphila*, Fatty acid profile, Halotolerant bacteria.

Introduction

Microorganisms able to sense environmental parameters that define their habitats and that they respond to stresses by many ways to vary their cell physiological processors in a suggestion to adapt to new environmental stress. Salinity stress could be a common type of abiotic stress that limits microbial growth. Microbial adaptation to salinity is one amongst the foremost interesting domains of microorganisms. Halophilic and halotolerant microorganisms are metabolically different and are adapted to extreme salinity. The bacterial response to salinity might include morphological, physiological and biochemical changes, and induced defensive mechanisms (Rothschild & Mancinelli, 2001; DasSarma & Aora, 2002 and Le Borgne et al., 2008). Different microorganisms have the ability to adapt or tolerate the salinity stress by many ways such accumulating osmolytes (Zahran et al., 1992 and Sagot et al., 2010), amino acid substitutions (Lanyi, 1974) and cell membrane lipids modification (Kaneda, 1991 and Vargas et al., 2005). Especially, an important adaptive response of bacterial cells to salt stress is that the modifications in their fatty acids and lipids (Ventosa et al., 1998; Nicolaus et al., 2001; Kaye & Baross, 2004 and De Carvalho et al., 2014). In most microbial cells fatty acids occur in their free form in small amounts. While they are built-up to form complex lipids in plasma membrane structure, energy storage, sign functions and as metabolic precursors (Pappas et al., 2004; Dibrova et al., 2014 and Sohlenkamp & Geiger, 2016). They are also constituents the lipid A part of lipopolysaccharides of the outer membrane of Gram-negative bacteria (Raetz et al., 2007). Membranous fatty acid in bacteria are for the most part varied depending on environmental conditions (Guerzoni et al., 2001; Van de Guchte et al., 2002 and Kimoto-Nira et al., 2009).

Indeed, each state of thinness and integrity of bacterial membranes are necessary to substitute some necessary functions such as the maintenance of a proton-motive force or the active transport of metabolites (Russell, 2002). The aim of this study was to evaluate the microbial growth

Corresponding author email: halmouch@yahoo.com Tel: +20 1068674884, Fax: +2057403868; Cell phone: 01285696169.

DOI: 10.21608/ejbo.2019.7553.1282

Edited by: Prof. Dr. Salama A. Ouf, Faculty of Science, Cairo University, Giza 12613, Egypt.

of *Halomonas alkaliphila* YHSA35 cells to a different concentration of NaCl and to research their salinity adaptation by sorting out the modification in cellular fatty acid profiles as a resistant mechanism to salt stress in *Halomonas alkaliphila* YHSA35.

Materials and Methods

Bacterial strain and growth conditions

H. alkaliphila YHSA35 is an interesting halotolerant and alkaliphilic Gram-negative bacterium. It was previously identified by El-Halmouch & Amer (2016) using phenotypic, biochemical and molecular biology analysis. YHSA35 strain grows well aerobically in nutrient broth medium supplemented with NaCl at an optimum temperature of 35°C and optimum pH at 8.5.

Preparation of bacterial inoculum

The bacterial strain of *H. alkaliphila* YHSA35 was maintained in 50% (v/v) glycerol at -20°C was re-cultivated in nutrient broth for 24h at 35°C. The bacterial cells were harvested by centrifugation, (25°C, 4000xg, 5min), washed using phosphate-buffered saline with 0.85% (w/v) NaCl, then re-suspended and diluted with deionized sterilized water to obtain a low concentration of 10^5 (cell/ml) before being used as bacterial inoculum.300µm was used to inoculate 100ml of liquid nutrient broth culture medium in 250ml flasks.

UV–vis scanning

Bacterial growth was estimated spectroscopically using Ultraviolet-visible (UV–vis) spectrophotometer ((JASCO V-630). 1ml bacterial culture of three days old was sediment at 5000r/min for 10min, the supernatant was discarded whereas sediment was suspended in 1ml distilled water. UV-vis scan (250–750nm) was recorded at room temperature. Changing in bacterial growth was determined at OD_{600nm} (Lauth et al., 2002) and at OD_{750nm} (Huang et al., 2002). Changing DNA to protein was monitoring using OD_{260nm}/OD_{280nm} ratio (Wang et al., 1995; Piereira et al., 2000 and Alupoaei & García-Rubio, 2005).

Lipid extraction and determination

The growing bacterial cells of three days old were harvested by centrifugation at 10,000rpm for 20min. The bacterial extraction was done according to Bligh and Dyer method (Bligh & Dyer, 1959) with some modifications. Known amount of biomass (100mg) was then homogenized in 100ml of chloroform: methanol (2:1 v/v) and extracted for 2h at 55ºC under a reflux condenser, extraction of the residual lipids was repeated with an additional 50ml of chloroform: methanol mixture and therefore supernatants were collected in a separating funnel. Then the lipid phase was separated by adjusting the solution ratios to 10:10:9 by volume (methanol: chloroform: water) by adding pure methanol and water to the solution. The lower layer of chloroform was separated and treated with anhydrous Na_2SO_4 to get rid of the traces of water. The lipid content was determined gravimetrically and expressed as dry weight percentage after evaporating the chloroform.

Preparation of fatty acid methyl esters

Fatty acid methyl esters (FAMEs) from the extracted lipids of *H. alkaliphila* YHSA35 were prepared as described by Radwan (1978). A sample of lipids was trans-esterified by the addition of 4ml benzene and 20ml 1% sulfuric acid in absolute methanol. The mixture was left refluxed at 90°C for 90min, then 20ml distilled water was added and thereforeresulted esters were extracted with 10ml benzene upon separation of the layers. The benzene layer was dried by using anhydrous sodium sulfate. Then the solvent was evaporated using rotary evaporator. The composition of FAMEs was quantified and identified using GC-MS.

GC analysis of fatty acid methyl esters

The total Fatty acids composition of lipids was analyzed as methyl esters with a gas chromatograph mass-spectrometer (Agilent 7890A GC system, USA) equipped with a capillary column (HP-5MS, length 30m, diameter 0.25mm). Chromatographic conditions were: carrier gas, helium; flow rate, 1.5 ml/min; sample input temperature, 290°C; initial temperature, 90°C for 1min, programmed to 300°C at the rate of 8°C; detector temperature, 300°C. FAs were identified by mass spectra and were compared for retention times with those of standards (“Sigma”, USA).

Statistical analysis

Data were subjected to one-way ANOVA and also the means were compared according to the Student–Newman–Keuls (SNK) multiple range test ($P \leq 0.05$).

Results

Growth pattern

The optical density of diluted aliquot samples of the whole medium broth was measured over the entire UV-Vis wavelength spectrum (250-750nm) against bacterial free culture medium (Fig. 1). An investigated strain was capable to grow in either the absence or presence of NaCl with a remarkable variation in growth pattern. As shown in Fig. 1, the growth of *H. alkaliphila* YHSA35 varied considerably in response to NaCl concentration. The strain YHSA35 tolerated NaCl up to 1800mM and showed optimum growth at 600mM (Figs. 1, 2). Bacterial absorbance at 600nm was slightly largerthan thatof 750nm, however that they had comparatively the same pattern (Fig. 2). Insignificant variations weredetermined for the growth at 600, 800 and 1000mM NaCl.

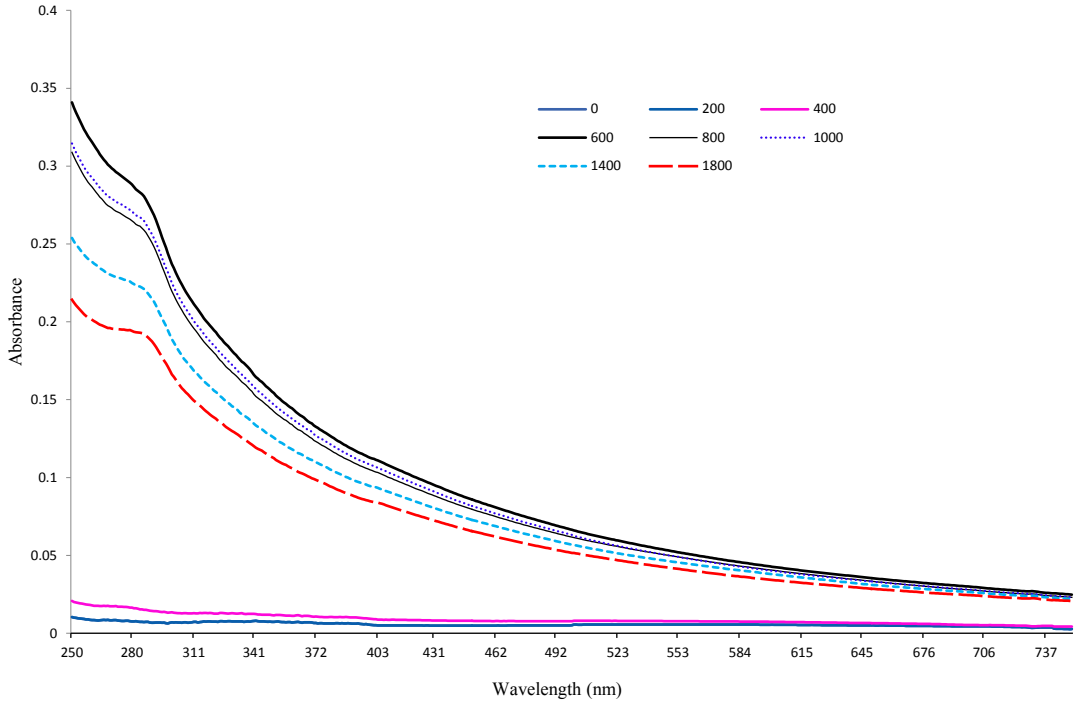

Fig.1. UV-vis spectra (from 250 to 750nm) for *H. alkaliphila* YHSA 35 grown at different NaCl concentrations.

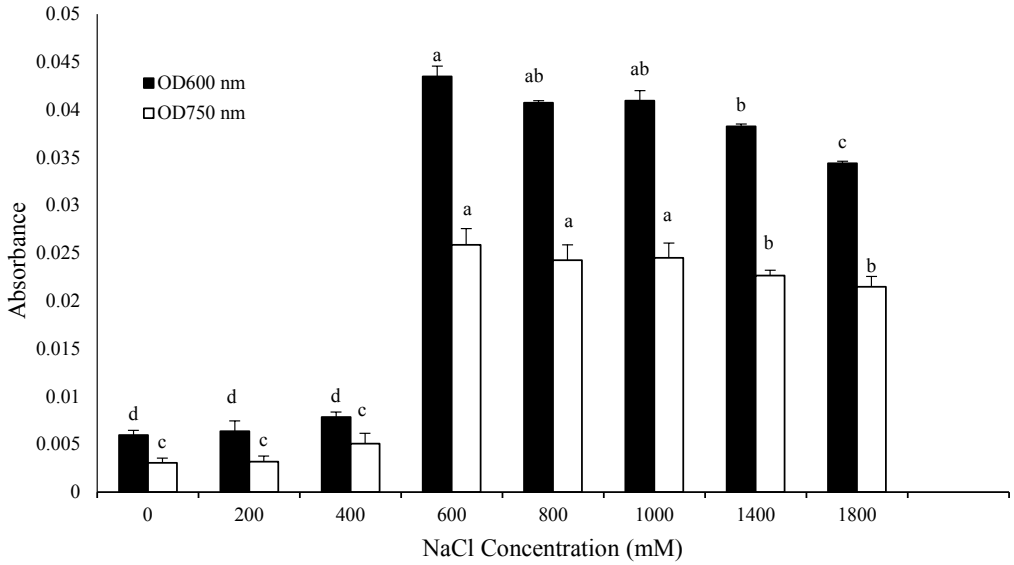

Fig. 2. Absorbance of *H. alkaliphila* YHSA35 culture grown at different salt concentrations [Different letters on the column for each parameter (600nm and 750nm) differ significantly at P≤0.05].

A_{280}/A_{260} ratio

UV-Vis absorption spectral analysis of various cultivation conditions exhibited several variations. These were clear in UV region (250-400nm), wherever A_{280}/A_{260} ratio varied significantly in line with the salt concentration (Fig. 3). As shown in Fig. 3, the ratio of OD_{280}/OD_{260} was always less than 1. A dramatic increase was determined with increasing the salt concentration over 600mM. The best ratio was determined at 1800mM (Fig. 3).

Fatty acids profile

The fatty acids profile and total fatty acids content of *H. alkaliphila* YHSA35 grown at different levels of salinity were compared at three different concentrations (0.0, 600 and 1800mM) (Fig. 4). Fatty acids methyl esters (FAME) composition analysis showed the presence of all the tested thirty-four fatty acids in *H. alkaliphila* YHSA35 (Table 1). At free salt medium, strain YHSA35 contains remarkably high proportions of unsaturated fatty acids (56% of the whole fatty acids and most common being C18:1). Cells grown up on 600mM NaCl showed the very best fatty acids content, 104.5mg/L (Table 1). Bacterial cells grown in 1800mM NaCl showed the very best saturated fatty acid content, 50.7mg/L, compared to alternative treatments. In contrast, the very best unsaturated fatty acids content, 75.7mg/L, was determined in case of cells grown at 600 mM NaCl, whereas cells grown up at 0.0 and 1800mM showed low unsaturated fatty acids content 49.3 and 46mg/L, respectively (Table 1).

It is determined that the even-chain fatty acids of each saturated and unsaturated were markedly affected by the salt concentration than the odd-chain (Table 1). For saturated fatty acids, Palmitic acid (16:0) was the foremost abundant in all salt treatments, followed by Stearic (18:0), Lignoceric acid (24:0) and Caprylic acid (8:0). Whereas the others were considered as a minor saturated fatty acid, they represented in a very low concentration ranging from 0.158 to 2.135mg/L. Regarding unsaturated fatty acids, Oleic (18:1), cis-11-Eicosenoic (20:1) and Erucic acids (22:1) were the foremost superabundant fatty acids (Table 1).

Although Myristic acid (14:0) and Lauric acid (12:0) gradually decreased in response to salt concentration, 0, 600 and 1800mM NaCl; recorded data of cells grownup at 600mM exhibited low concentration of Palmitic acid (16:0), 8.9mg/L, that was nearly double, 18.7mg/L, just in case of cells grown at free salt medium, whereas just in case of cells grown up at 1800mM exhibited near fourfold, 34.7mg/L, above that of cells grownup at 600mM NaCl (Fig. 5).

In contrast, a dramatic increase was determined in three unsaturated fatty acids once the salt was accumulated, with a peak at 600mM NaCl. Wherever Oleic acid (18:1) (Fig. 5), cis-11-Eicosenoic acid (20:1) (Fig. 5) and Erucic acid (22:1) showed the very best content simply just in case of cells grown up at 600mM compared to cells grown at 0 and 1800mM (Table 1). They represent about 51.4%, 68.5% and 46.1% of the unsaturated fatty acids (USFA) content at 0.0, 600 and 1800mM NaCl, respectively. The ratio of monounsaturated fatty acids (MUFA) to polyunsaturated fatty acids (PUFA) varied among the bacterial cells in line with the tested salt concentration. It was 2.64, 4.09 and 1.99 in cells grown up at 0, 600 and 1800mM NaCl, respectively.

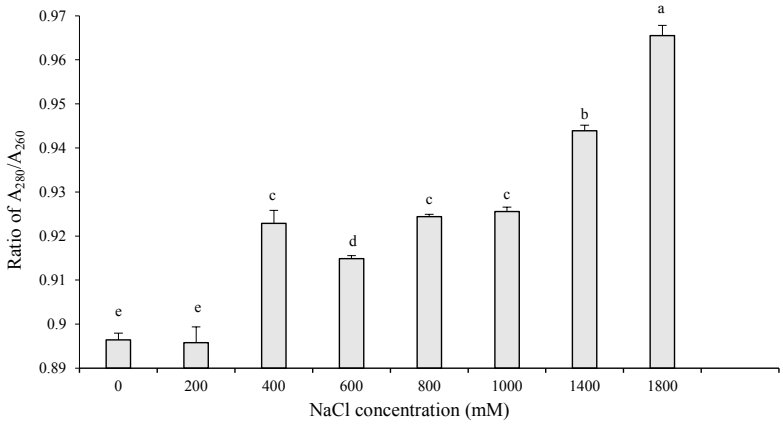

Fig. 3. A_{280}/A_{260} ratio of *H. alkaliphila* YHSA35 culture grown at different salt concentrations [Different letters differ significantly at $P \leq 0.05$].

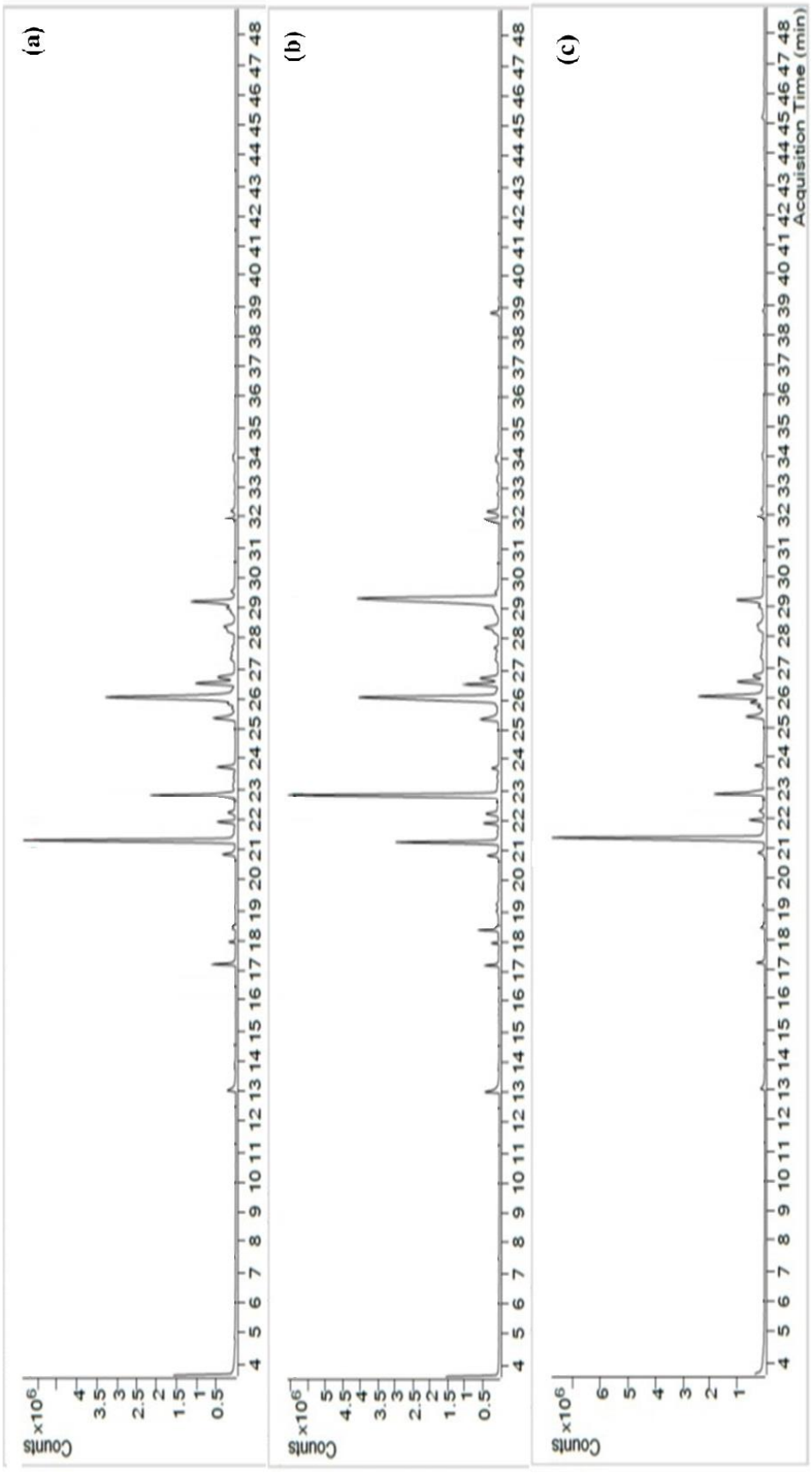

Fig. 4. Representative GC-MS chromatogram of fatty acid methyl esters (FAMEs) of *H. alkaliphila* YHSA35 grown at different concentrations of sodium chloride [(a), 0; (b), 600 and (c), 1800mM].

TABLE 1. Fatty acids profile and their quantity (mg/L) of *H. alkaliphila* YHSA35 grown at different NaCl concentrations.

Fatty acids	Station	AT	Salt concentration (mM)		
		min	0.0	600	1800
Caproic	6:0	3.91	0.736	2.023	0.158
Caprylic	8:0	7.17	2.269	2.257	2.270
Capric	10:0	10.59	0.383	0.337	0.298
Undecanoic	11:0	12.22	0.375	0.444	0.225
Lauric	12:0	13.90	0.751	0.482	0.376
Tridecanoic	13:0	15.54	0.366	0.321	0.312
Myristoleic	14:1	16.92	0.728	0.716	0.662
Myristic	14:0	17.22	1.893	1.433	1.176
cis-10-Pentadecenoic	15:1	19.09	0.738	0.726	0.751
Pentadecanoic	15:1	19.12	0.781	0.532	0.915
Palmitoleic	16:1	20.86	3.028	3.070	2.622
Palmitic	16:0	21.31	18.738	8.938	34.726
cis-10-Heptadecenoic	17:1	23.38	1.091	0.978	1.024
Heptadecanoic	17:0	23.74	1.518	0.994	1.292
gama-Linolenic	18:3	25.71	1.708	1.648	1.659
Linolenic	18:2	26.07	1.634	1.613	1.598
Oleic	18:1	26.07	17.857	23.197	12.105
Elaidic	18:1	26.22	1.038	1.030	1.041
Stearic	18:0	26.53	3.104	3.039	3.053
Arachidonic	20:4	31.40	1.712	1.758	1.869
cis-5,8,11,14,17-Eicosapentaenoic	20:5	31.11	1.618	1.498	1.563
cis-8,11,14-Eicosatrienoic	20:3	31.86	1.662	1.688	1.668
cis-11,14-Eicosadienoic	20:2	32.24	1.532	1.580	1.523
cis-11-Eicosenoic	20:1	32.24	2.027	4.001	1.864
cis-11,14,17-Eicosatrienoic	20:3	32.20	1.582	1.686	1.530
Arachidic	20:0	32.82	1.043	1.307	0.992
Heneicosanoic	21:0	36.27	1.141	1.153	1.117
cis-4,7,10,13,16,19-Docosahexaenoic	22:6	37.37	1.633	1.666	1.607
cis-13,16-Docosadienoic	22:2	38.82	1.762	1.869	1.764
Erucic	22:1	38.80	5.465	24.634	7.237
Behenoic	22:0	39.29	2.091	2.181	2.135
Tricosanoic	23:0	41.90	1.298	1.294	1.318
Nervonic	24:1	43.86	1.687	1.681	1.684
Lignoceric	24:0	44.19	2.588	2.579	2.568
Saturated			38.3	28.8	50.7
Unsaturated			49.3	75.6	46
Total			87.6	104.4	96.7

AT: Acquisition time.

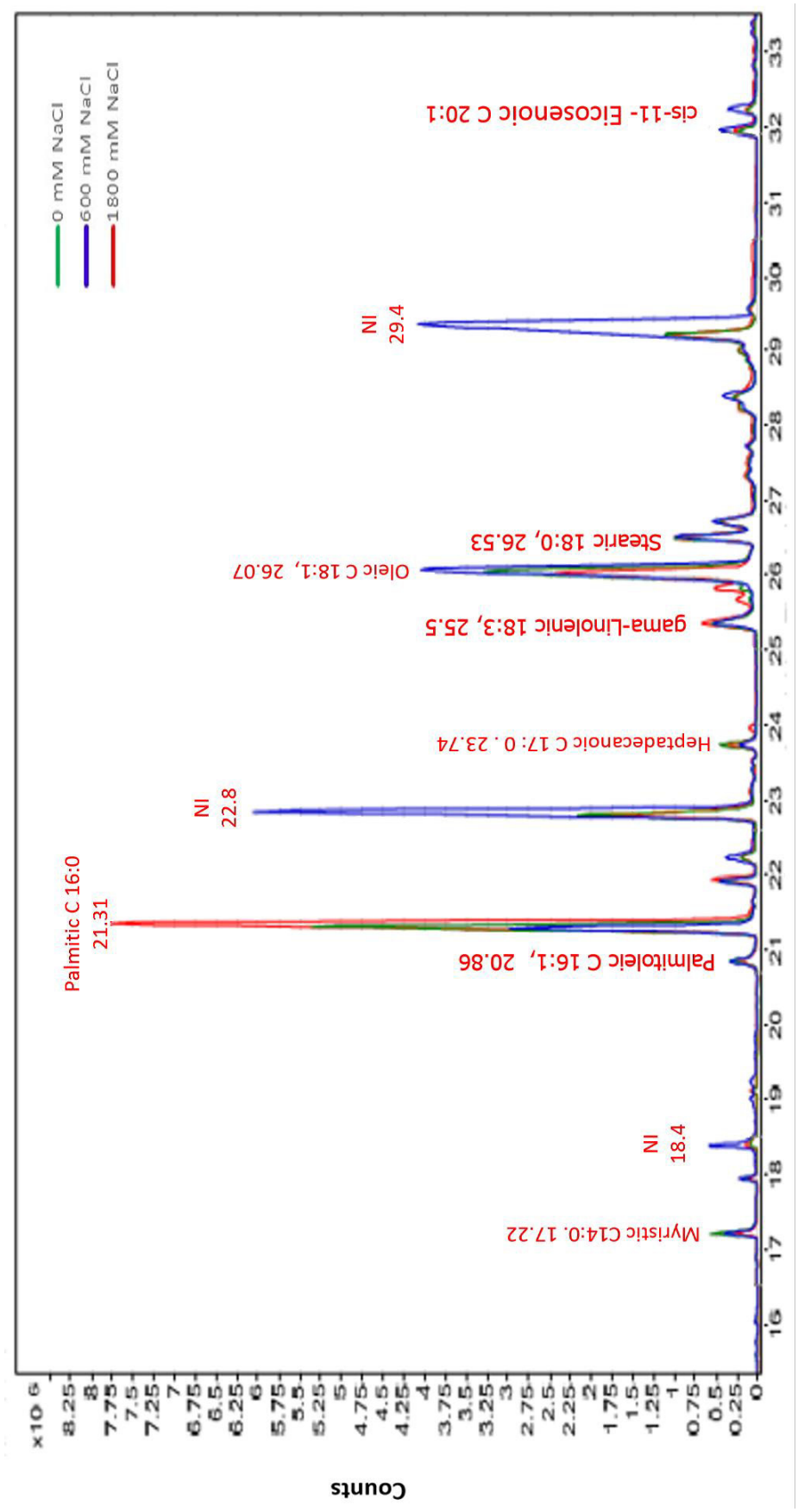

Fig. 5. Comparison btween the most variable fatty acids profiles of *H. alkaliphila* YHSA35 in response to sodium chloride concentration [NI: not identified fatty acid].

Discussion

Exposure of microorganisms to high osmolality environment triggers rapid fluxes of cell water along the osmotic gradient out of the cell, so causing a reduction in turgor and dehydration of the cytoplasm. Bacterial adaptation to salinity involves a rise in intracellular solute pool by accumulation of ions and/or organic osmolytes (Galinski & Tròper, 1994; Galinski, 1995 and Long, 2018) and structural changes within the cell envelope (Guérin-Méchin et al., 1999; Dubois-Brissonnet et al., 2001; Chládková et al., 2004 and Dubničková et al., 2006), within the plasma membrane and a serious re-programming of the cell metabolism (Paul et al., 2005; Nabarlatz et al., 2010 and Cortés-Lorenzo et al., 2012). Inorganic salt has an important role in the microbial growth. Like different bacteria, *H. alkaliphila* YHSA35 growth varied instep with the salt concentration in its culture medium. Our results showed that the proper quantity of NaCl salt for *H. alkaliphila* YHSA35 growth was 600mM NaCl. It will promote the metabolism of microorganisms. The gradual increase in NaCl concentration had a negative result on the bacterial growth. Moreover, the high level of NaCl inhibits the growth of the microorganism. The explanation is also that high salinity will interfere with the normal metabolic pathways of microorganism, destroys microbial cell membrane (Russell et al., 1995 and Elkahoui et al., 2004), reduces the enzyme activity and/or produces new enzyme system (Cortés-Lorenzo et al., 2012 and He et al., 2017). A special biosynthesis pathway for proline production under salt stress conditions has been detected in *Bacillus subtilis* by Belitsky et al. (2001).

In the initial part of this work, we have got shown that increasing salinity in the culture medium causes a disturbance in protein/nucleic acid ratio. The raising of OD_{280}/OD_{260} ratio offers a strong proof to cell modifications in the protein content to adapt the salinity. In this discipline, it is going to be aforementioned that salinity will promote or inhibit several enzyme reactions in the microbial cell. This might result in a change in one/ or some metabolic pathways in the cell. This suggestion was confirmed by several workers like Gatti et al. (2010), Nabarlatz et al. (2010) and Hong et al. (2013).

This research curious about looking for some changes in fatty acids content under the salt stress. Lipids, particularly fatty acids are the foremost effective source of stored energy and have important role in tolerance to several physiological stressors in all organisms including bacteria. A modification in total fatty acids content was observed when *H. alkaliphila* was grown in salt replete condition. Analytical knowledge from many authors (Xu & Beardall, 1997; Guerzoni et al., 2001; Turk et al., 2004 and Al-Khalaf et al., 2012) have shown that the salinity can have a serious influence on the fatty acid composition of bacteria. Variety of researchers have investigated that the microbial cells can defend themselves against salt stress by lipid accumulation (Huflejt et al., 1990 and Khomutov et al., 1990).

Indeed, fatty acids are precursors for a spread of necessary building compounds (attach to proteins in plasma membrane), secondary metabolites and signaling molecules. Bacterial cell envelope is the first line of defense against all the environmental stress factors. Therefore, the majority of bacterial cell-extracted fatty acids of Gram-negative bacteria originate from the membranes. Alterations within the fatty acids profile might alter membrane permeability and fluidity, in turn might contribute to salinity tolerance. Fatty acids are the most variable part in phospholipids to challenge these environmental stresses. They are ester-linked to a glycerol backbone in the plasma membrane (Alvarez & Steinbuüchel, 2002; Lu et al., 2004 and Sohlenkamp & Geiger, 2016). The number and position of double bonds of acyl chains by specific fatty acid desaturases plays a crucial role in preserving a suitable dynamic state of the bilayer under the environmental stress (Šajbidor, 1997).

This work suggested that the salinity includes a nice influence on the saturation degree of intracellular fatty acids within the vegetative cells. Growth at sublethal concentrations of salt led to a better saturation of the fatty acids and vice versa. These agree with those obtained by several workers. Wherever they investigated that the microbial growth under biotic and/or abiotic stress has a direct influence in saturated and unsaturated fatty acids degree among the membrane (Sullivan et al., 1979; Arneborg et al., 1993; Rozes & Peres, 1998 and Shin et al., 2018). Many authors reported that an increase in fatty acids saturation degree afterwards decrease the membrane fluidity (Keweloh et al., 1991; Weber & de Bont, 1996 and Fakhruddin & Quilty, 2006).

In this work, the bacterial growing at 600 and 1800mM NaCl concentration showed a significant

variation in each saturated and unsaturated fatty acids compared to the salt-free. It is reportable that the total unsaturated fatty acid content decreases once the osmolality of the growth medium is enhanced. Increasing salt concentration over the optimum induced the decrease of monounsaturated fatty acid (C18:1). These results consider several works achieved by Banciu et al. (2005) in *Thioalkalivibrio* spp., by Valderrama et al. (1998) in *Halomonas* spp. (*H. salina* and *H. halophila*) and Gilarova et al. (1994) in *Lactobacillus* strains. These results were additionally confirmed by Valderrama et al. (1998) who reported an increase in cyclopropane fatty acids with a decrease in monounsaturated fatty acids in *Halomonas salina*, when grown at different salt concentrations. Our results recommended that, unsaturated fatty acids mightplay a vital role in increasing the fluidity of the bacterial membranes of *H. alkaliphila* YHSA35 growing at optimum salt concentration (600mM NaCl). Previous suggestion was confirmed by Mutnuri et al. (2005) who declared that increasing the concentration of unsaturated fatty acids at optimum salt concentration makes the membrane more flexible, avoiding bursting of cells due to increased influx of water.

In the same context, Dubničková et al.(2006) recommended that increasing unsaturation reflect the importance of maintaining the optimum outer membrane fluidity and stability in *Escherichia coli* ATCC 11229 under adverse conditions.

The results showed that monounsaturated fatty acids especially, oleic, cis-11- eicosenoic and erucic play a vital role in membrane fluidity regulation. They probably reduce the ions permeability of the bacterial cell. This suggestion in agreement with Fozo & Quivey (2004), who assumed that increasing in monounsaturated fatty acids in *Streptococcus mutans* reduce cells' permeability to protons in an acidic environment.

On the contrary, microbial growth at non-optimal salt concentrations will increase the concentration of palmitic acid as the saturated fatty acid. Accumulation of saturated fatty acids in the bacterial membrane can result in a significant disturbance of physiological function and cell death. These are in accordance with those of Denich et al. (2003) who reported that the saturation of the membranous fatty acids is that the reason why membrane flexibility and adaptation ability verify the survival of the cell. In further, fatty acids variations under salinity stress are going to be useful in improvement of those bacterial cells in their bioremediation, bacterial achievement to produce certain fatty acids, looking for various enzymatic reactions and their regulation involved within the biosynthesis of these fatty acids and understanding the mechanisms of fatty acids defense against the salinity.

Acknowledgments: I thank Dr. Eithar El-Mohsnawy and Dr. Abdelhamid El-Shaer for laboratory help and helpful discussion throughout the study. Thanks, are also due to Prof. Dr. Abd El-Raheem R. El Shanshoury, Professor of Microbiology, Tanta University for revising the manuscript.

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(*Received* 23/1/2019;
accepted 31/3/2019)

التغيرات التكيفية في الأحماض الدهنية المشبعة كألية مقاومة للإجهاد الملحي في بكتريا *Halomonas alkaliphila* YHSA35

ياسر الحلموش
قسم النبات والميكروبيولوجي – كلية العلوم – جامعة دمنهور – دمنهور – مصر.

تعد الملوحة من العوامل غير البيولوجية المجهدة، وتستطيع البكتريا مقاومة هذا الإجهاد الملحي بوسائل مقاومة عديدة. في البداية تمت دراسة وتتبع نمو بكتريا هالوموناس ألكاليفيلا (*Halomonas alkaliphila* YHSA35) تحت تأثير تركيزات مختلفة من كلوريد الصوديوم بواسطة مقياس الطيف الضوئي عديد الأطوال الموجية (UV-vis). أعطت التغيرات في نسبة الأمتصاص الضوئي للنموات البكتيرية عند الطولين الموجيين 260nm و 280nm انطباعا عن وجود تغيرات فسيولوجية تحاول بها البكتريا التكيف مع التغيرات في الإجهاد. في هذا العمل تم دراسة التغيرات الكمية لعدد أربعة وثلاثون من الأحماض الدهنية في البكتريا قيد الدراسة والتي لها القدرة على التكيف للاجهاد الملحي باستخدام جهاز الكروماتوجرافي (GC-MS)، و أوضحت النتائج أن هناك تغيرات كمية في كل من الأحماض الدهنية المشبعة مثل الكابرويك (Caproic)، ليريك (Lauric)، أندوكانويك (Undecanoic)، الميرستيك (Myristic)، البالميتك (Palmitic) و الهيبتاديكانويك (Heptadecanoic)، والأحماض غير المشبعة مثل الأوليك (Oleic)، ايكوسينويك (cis-11-Eicosenoic) و ايروكيك (Erucic). إن زيادة نسبة الملح في البيئة الخارجية تقلل من تكوين الأحماض الدهنية غير المشبعة وتؤدى إلى تراكم الدهون المشبعة في الخلية و أكثر هذه الأحماض هو حامض البالميتك (Palmitic) فهو يعتبر البادئ لبناء العديد من الأحماض الدهنية. ومن هذا المنطلق نستطيع القول بأن الأحماض الدهنية ربما تتدخل لتتحكم في تركيب وضبط درجة لزوجة الدهون في الغشاء البلازمي وتكيف بروتوبلازم الخلية لتقاوم زيادة الإجهاد الملحي.