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GC-MS ANALYSIS OF CHANGE IN FATTY ACID COMPOSITION OF HALOBACTERIUM *BACILLUS LICHENIFORMIS* HSW-16 UNDER VARYING SALINITY CONDITION

Rajnish Prakash Singh[1]*, *Somesh Mishra*[2], *Smita Raghuvanshi*[2], *Prabhat Nath Jha*[1]

Address(es): Rajnish Prakash Singh,
[1]Dept. of Biological Sciences, Birla Institute of Technology and Science (BITS), Pilani-333031, Rajasthan, India.
[2]Dept. of Chemical Engineering, Birla Institute of Technology and Science (BITS), Pilani-333031, Rajasthan, India.

*Corresponding author: manasrajnish2008@gmail.com

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ABSTRACT

The salt concentration have a pronounced effect on fatty acid composition, therefore the adaption of fatty acid composition of a halophilic bacterium *Bacillus licheniformis* HSW-16, grown at different concentration of NaCl (0 mM, 150 mM, 175 mM, 200 mM) was studied. The common fatty acid observed at various salt concentration were tetratriacontane, triacontane, tetracosane and pentacosane etc..As the salinity increases from 150 mM to 200 mM, the bacterium started to accumulating the long chain alkanes and fatty alcohol, suggesting the adaption of bacterium through accumulation varying fatty acids for counteracting the increased salinity and maintaining its osmotic balance in the changing environment.

Keywords: *Bacillus*, fatty acid, *Halobacterium*, salinity stress, Salt lake

INTRODUCTION

Halophilic organism is able to survive under hypersaline and alkaline conditions. The natural niche for these microorganismsis saline soil, salt lake, soda lake etc. (**Oren, 2002**). However salt tolerant bacteria has been divided into two groups, one euryhaline: can be able to grow without salt, and stenohaline: NaCl compulsory for growth and survival (**Vreeland, 1987**). To cope with changing environmental stresses, these microorganisms accumulate specialized molecules and complexed physiological mechanism. As the salinity increases it imposed the physiological drought stress conditions on a living microorganism that change the osmotic pressure also. Therefore, to protect the cells under these adverse environmental conditions, creation of a hydrophobic membrane layer would be more advantageous. Previous study suggested that under changing environment, the total unsaturated fatty acid content decreases when the osmolality of the growth medium is increased **Miller KJ, (1986)**.The salinity and alkalinity of the soil is increasing day by day because of the extensive use of chemical fertilizer, pesticides etc. for increasing the agricultural productivity and yield.Also, the growth of crop and its productivity is affected by increased salinity therefore, utilization of haloalkalophilic bacterium for reducing the effect of salinity and alkalinity has drawn considerable attention to promote plant growth (**Mishra *et al.*, 2015**). The present study aimed to study the fatty acid analysis of a halophilic bacterium *Bacillus licheniformis*HSW-16 to a wide range of salt stress conditions.

MATERIAL AND METHODS

Bacterial strain and culture condition

A halotolerant bacterium *Bacillus licheniformis* HSW-16 was isolated from the hypersaline lake water of Sambhar lake. Previous study hasreported that thesalinity of lake is 7% (w/v) with salt concentration in the range of 12% to 30% (w/v). The sulphates, carbonates, bicarbonates, chlorides, sodium and smaller amounts of potassium salts are the major contributor to the salinity (Joshi and Seth 2008). Isolated strain was capable to grow in presence of NaCl up to concentration of 2 to 11% (w/v). To studying the change in fatty acid composition of strain HSW-16, it was cultured in DF medium supplemented with salt concentration (0 mM, 150 mM, 175 mM, 200mM).Composition of DF medium was as follows (per litre): KH_2PO_4 4.0g, Na_2HPO_4 6.0g, $MgSO_4.7H_2O$ 0.2g, glucose 2.0g, gluconic acid 2.0g, citric acid 2.0g, Trace elements: $FeSO_4.7H_2O$ 1mg, H_3BO_3 10µg, $MnSO_4.H_2O$ 11.19µg, $ZnSO_4.7H_2O$124.6µg, $CuSO_4.5H_2O$ 78.22µg, $MoO_3$10µg, pH 7.2 (**Dworkin and Foster, 1958**).

Lipid extraction

The bacterial strain HSW-16 was grown in DF medium supplemented with different salt (NaCl) concentration from 150 mM to 200 mM and incubated for 24 h in a rotator shaker at 30 °C. After the incubation period culture was centrifuged at 10,000 g for 20 min at 4 °C. After centrifugation, the supernatant was discarded and cell pellet was retained for fatty acid profiling. The obtained cell pallet was suspended in sonicated buffer containing Tris–HCl (50 mM, pH 7.6), dithithretiol (1.l M), 1 mMphenylmethylsulfonyl fluoride (PMSF) and lysozyme (0.2%) and sonication was carried out to obtain the cell lysate. The cellular lipids were extracted in the solvent system, chloroform and methanol in the ratio of 2:1. The obtained cell lysate was diluted ten times with the solvent system and kept on a shaker at 160 rpm for 3 h to separate the phases. The lower organic phase was concentrated on rotary evaporator and re-dissolved in toluene and converted into fatty acid methyl ester (FAME) by transesterification reaction as described in preceding section.

Transesterification and FAME analysis

Transesterification reaction was carried out by as per the method of **chriestie, (2003)**. The extracted cellular fatty acids, dissolved in toluene (5mL), were subjected to base catalyzed transesterification reaction. One molar (1 M), 100 ml stock solution of sodium methoxide was prepared by adding 2.3 g of sodium metal in methanol. In a 250 ml conical flask, sodium methoxide and methanol solution was taken in the ratio 1:2 and 2 ml of extracted fatty acids dissolved in toluene was added to it. The flask was sealed with a cotton plug to prevent from any contamination and kept in the oven at 50 °C for 2 h. After 2 h, the glacial acetic acid of 0.1 mL was added followed by 5 mL distilled water. The resulting esters were extracted in 10 ml of hexane analytical grade. The hexane layer was dried over anhydrous Na_2SO_4 to remove the trace of water. The hexane phase was concentratedon a rotary evaporator. The final concentrated hexane phase rich in esters were profiled using GC-MS (Shimadzu QP-2010 Plus).

Fatty acid analysis by gas chromatography (GC)

The profiling of resultant FAME samples (l µL) was carried out using gas chromatography-mass spectroscopy (GC-MS) (Shimadzu QP-2010 Plus) equipped with a split/splitless injector and capillary column DB-5 MS (0.25 µm film thickness, 0.25 mm i.d., 30 m length). The conditions applied for GC analysis were: splitless mode, initial oven temperature 50 °C which was held for 2 min, injector temperature 250 °C and detector temperature 230 °C. The

temperature was increased from 50 °C to 250 °C at a rate of 10 °C per min and was held for 3 min on reaching 250 °C. The temperature was further increased from 250 °C to 280 °C at a rate of 15 °C per min and was held for 5 min on reaching 280 °C. The helium of ultra-high purity was utilized as carrier gas (head pressure was 72.5 kPa and flow rate was maintained as 1.21 ml min^{-1}). The conditions applied for MS analysis were: scan mode, start time 4.00 min, end time 31.99 min, scan speed 1250, event time 0.5 second, start m/z 40.00 and end m/z 650.00. Data was compared with the inbuilt standard mass spectra library system (NIST-05 and Wiley-8) of GC–MS.

RESULTS AND DISCUSSION

The proficiency of HSW-16 in the environment of varying salt concentration (150 mM to 200 mM) was studied by observing the composition of cellular extract. The composition of cellular extract was analyzed using GC-MS analysis at respective salt concentration. A control was also placed with 0 mM salt concentration to investigate the change in fatty acid composition. The obtained GC-MS profile of cellular extract at different salt composition were comprised of hydrocarbons, fatty alcohols and fatty acids with carbon chain length varying in range of C_{13}-C_{44}. At 0 mM salt concentration (Table 1.), the composition of extract was mainly hydrocarbons with n-tetracosane (6.91%), n-tetracontane (9.26%), hexatriacontane (11.16%), pentacosane (10.72%), n-tetratriacontane (9.34%), n-tetratetracontane (7.34%). The GC-MS analysis of extract at 0 mM salt concentration also showed the presence of fatty acids like tetradecanoic acid (0.47%), octadec-9-enoic acid (0.90%), and pentadecanoic acid (2.8 %). As the salt concentration increases from 0 mM to 150 mM (Table 2.), the extract composition changes with increase in concentration of long chain alkanes like hexatriacontane (11.16%), pentacosane (12.56%), n-tetratriacontane (12.15%), n-tetratetracontane (10.25%). The cell extract (Table 2.) also shows the presence of fatty alcohols like 1-heptadecanol (0.07%), 1-nonadecanol (0.13%), 1-tetracosanol (0.14%). At salt concentration of 175 mM (Table 3.), the composition of cell extract was mainly hydrocarbons like n-tetracosane (3.08 %), n-tetracontane (6.20%), hexatriacontane (11.20%), pentacosane (10.85%), n-triacontane (10.01%), n-tetratriacontane (6.88 %). Similar composition of cell extract was observed at salt concentration of 200 mM (Table 4.) having hydrocarbon like n-tetracosane (6.54%), n-tetracontane (9.14%), hexatriacontane (11.02%), pentacosane (10.69%), n-tetratetracontane (9.65%), n-tetratriacontane (7.56%). At 200 mM salt concentration, fatty alcohols like 1-heneicosanol (0.97 %), 1-heptadecanol (0.78%), 1-tetracosanol (0.51%) were also present in the intracellular cell extract. Thus, the GC-MS analysis of cellular extract at varying salt concentration confirmed the presence of long chain hydrocarbon, fatty acid and fatty alcohol. The analysis also shows that as the concentration of the salt increases in the cellular vicinity, the intracellular composition of the cell changes. The two strategies implemented by prokaryotes for survival in osmotic stress is 1: 'salt in cytoplasm' approach, which requires extensive structural modifications, is restricted mainly to members of the *Halobacteriaceae*, 2: accumulation of a restricted range of compatible solutes (Present work). In present work, in order to retort the osmotic stress, cell starts accumulating long chain alkanes and fatty alcohol which helps in maintaining the cellular membrane as iso-osmotic with changing salt concentration and crucial for bacterial cell survival. The bacterial species are capable of synthesize fatty alcohols and hydrocarbons intracellular as well extracellular having chain length ranging from C_9-C_{20}. The present study confirmed that the assimilation of different compounds of different composition, nature in response to changing environment of salinity was necessary for bacterium cell survival.

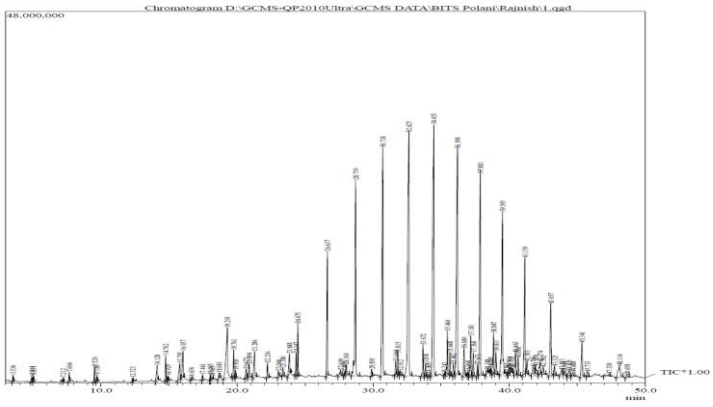

Figure 1 a) GC-MC chromatogram of HSW-16 at 0 mM NaCl

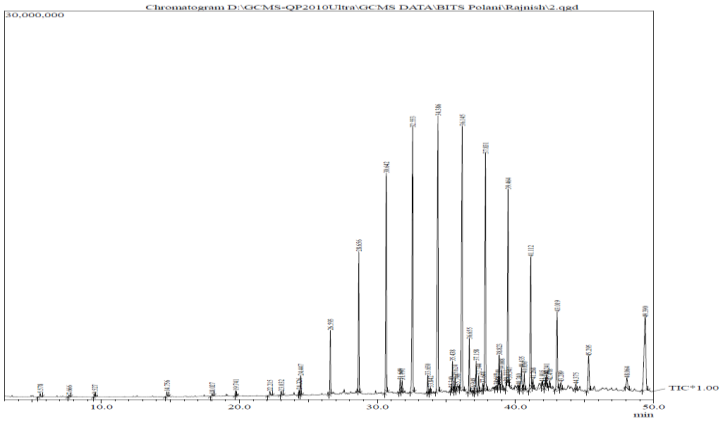

Figure 1 b) GC-MC chromatogram of HSW-16 at 150 mM NaCl

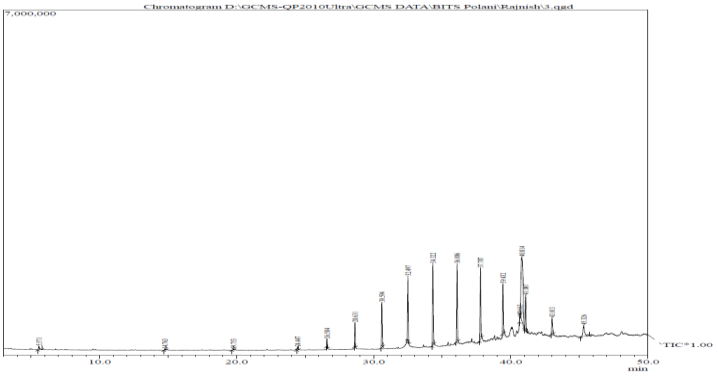

Figure 1 c) GC-MC chromatogram of HSW-16 at 175 mM NaCl

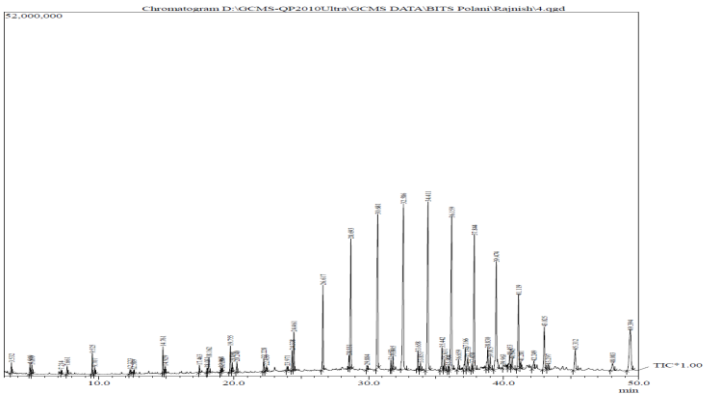

Figure 1 d) GC-MC chromatogram of HSW-16 at 200 mM NaCl

Table 1 GC-MS analysis on HSW-16 at 0mM salt concentration

S.No.	Run Time	Formula	Compound	% Area	Relative Molecular mass	Match quality %
1.	3.536	$C_{13}H_{28}$	Tridecane	0.12	184	96
2.	4.914	$C_{14}H_{28}$	Tetradecene-1	0.12	196	96
3.	14.128	$C_{14}H_{28}O_2$	Tetradecanoic acid	0.47	208	96
4.	16.037	$C_{18}H_{34}O_2$	Octadec-9-enoic acid	0.90	282	85
6.	19.291	$C_{15}H_{30}O_2$	Pentadecanoic acid	2.84	242	93
7.	26.637	$C_{30}H_{62}$	N-Triacontane	3.23	422	96
8.	28.719	$C_{24}H_{50}$	n-Tetracosane	6.91	338	96
9.	30.718	$C_{40}H_{82}$	N-Tetracontane	9.26	562	95
10.	32.625	$C_{36}H_{74}$	Hexatriacontane	11.16	506	95
11.	34.453	$C_{25}H_{52}$	Pentacosane	10.72	352	95
12.	36.198	$C_{34}H_{70}$	n-Tetratriacontane	9.34	478	95
13.	37.881	$C_{44}H_{90}$	n-Tetratetracontane	7.34	618	94
14.	44.044	$C_{27}H_{50}O_4$	Oxalic acid, cyclohexylmethyloctadecyl ester	0.14	438	84

Table 2 GC-MS analysis on HSW-16 at 150 mM salt concentration

S.No.	Run Time	Formula	Compound	% Area	Relative Molecular mass	Match quality %
1.	9.527	$C_{17}H_{36}O$	1-Heptadecanol	0.07	256	95
2.	14.756	$C_{19}H_{40}O$	1-Nonadecanol	0.13	284	96
3.	19.741	$C_{24}H_{50}O$	1-Tetracosanol	0.14	354	96
4.	26.593	$C_{25}H_{52}$	N-Triacontane	1.92	352	96
5.	28.656	$C_{24}H_{50}$	n-Tetracosane	4.65	338	95
6.	30.642	$C_{40}H_{82}$	N-Tetracontane	8.22	562	95
7.	32.553	$C_{36}H_{74}$	N-Hexatriacontane	11.20	506	95
8.	34.386	$C_{25}H_{52}$	Pentacosane	12.56	352	95
9.	36.145	$C_{34}H_{70}$	n-Tetratriacontane	12.15	478	95
10.	37.831	$C_{44}H_{90}$	n-Tetratetracontane	10.25	618	94

Table 3 GC-MS analysis on HSW-16 at 175 mM salt concentration

S.No.	Run Time	Formula	Compound	% Area	Relative Molecular mass	Match quality %
1.	28.631	$C_{24}H_{50}$	n-Tetracosane	3.08	338	95
2.	30.594	$C_{40}H_{82}$	N-Tetracontane	6.20	562	95
3.	32.497	$C_{29}H_{60}$	N-Nonacosane	8.58	408	95
4.	34.322	$C_{25}H_{52}$	Pentacosane	10.85	352	95
5.	36.086	$C_{36}H_{74}$	N-Hexatriacontane	11.20	506	94
6.	37.787	$C_{25}H_{52}$	N-Triacontane	10.01	352	96
7.	39.432	$C_{34}H_{70}$	n-Tetratriacontane	6.88	478	95

Table 4 GC-MS analysis on HSW-16 at 200 mM salt concentration

S.No.	Run Time	Formula	Compound	% Area	Relative Molecular mass	Match quality %
1.	19.755	$C_{21}H_{44}O$	1-Heneicosanol	0.97	312	96
2.	24.338	$C_{27}H_{56}O$	1-Heptacosanol	0.78	396	97
3.	26.617	$C_{30}H_{62}$	N-Triacontane	3.71	422	96
4.	28.551	$C_{24}H_{50}O$	n-Tetracosanol-1	0.51	354	85
5.	28.693	$C_{24}H_{50}$	Tetracosane	6.54	338	95
6.	30.681	$C_{40}H_{82}$	N-Tetracontane	9.14	562	95
7.	32.586	$C_{36}H_{74}$	N-Hexatriacontane	11.02	506	94
8.	34.411	$C_{25}H_{52}$	Pentacosane	10.69	352	95
9.	36.159	$C_{44}H_{90}$	n-Tetratetracontane	9.65	618	94
10.	36.659	$C_{30}H_{50}$	Squalene	0.41	410	93
11.	37.844	$C_{44}H_{90}$	n-Tetratriacontane	7.56	618	94

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