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FATTY ACIDS, HYDROCARBON TOLERANCE AND HEAVY METAL TOLERANCE PROPERTIES OF HALOALKALIPHILIC *Bacillus* sp.

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ABSTRACT

Microorganisms live almost everywhere including the harsh habitats on earth. In order to survive in these environments the microbes produce a variety of metabolite productcs and polymeric compounds which confer the ability to survive and thrive in such habitats. The haloalkaliphilic environments are harsher as they have a combination of saturating salinity and precipitating alkalinity, the bacteria like haloalkaliphilic *Bacillus sp.,* are specialized to thrive in these habitats. This is achieved through the production of different compounds including special fatty acids, which confer different properties to the bacteria. In this article the different ectoines produced was assayed by FAME (Fatty Acid Methyl Esters) analysis using GC-MS (Gas Chromatography-Mass Spectroscopy) and ability to tolerate hydrocarbons and heavy metals in the surroundings is measured for three haloalkaliphilic Bacillus sp., isolated from solar salterns. The FAME GC-MS assay revealed the ectoines based fatty acids are different in the three isolates and their quantity also varied among the isolates even the source of isolation was same. The results conclude the more the ectoines produced the more tolerance they showed to hydrocarbons (petrol, diesel, xylene, toluene, and kerosene) and heavy metals (mercury, lead, cadmium, chromium, aluminum, zinc, copper, and nickel). This is the imperative to understand, and even manipulate different bacteria to accumulate/and immobilize heavy metals and decrease the toxicity of hydrocarbons which are the major environmental pollutants.

KEYWORDS: Haloalkaliphilic Bacillus sp.; Fatty Acid Methyl Esters (FAME); Gas Chromatography-Mass Spectroscopy (GC-MS); Hydrocarbons; Heavy metals.

INTRODUCTION

Microorganisms are omnicompetent in survivability, and occupy and thrive in even the harshest of environments comprising the coldest, hottest, saltiest, the most acidic, and alkaline, which are supposed to be inhabitable based on human habitability criteria (Purohit *et al.,* 2014). In some instances the habitats have more than one restraining factor to survive with, such as halophilic milieu has acidity or alkalinity as a second limiting factor, in order to cope with these harsh and limiting environments microorganisms have evolved many distinct and diverse mechanisms making them unique from the rest of the organisms. One such group is haloalkaliphilic species which has the ability to cope with saturating salinity and acidity or alkalinity via., production of certain metabolites and proteins which reduce the stress on cytoplasm and more importantly the fatty acids produced are very effective in protecting the internal cation concentration thus giving rise to "keeping the salt out" strategy (Garabito *et al.,* 1998).

Along with the extracellular proton gradient based pumps to keep the internal ionic proportion the long chain fatty acids like Hentriacontane, Dodecane,1-Fluoro-, 2-T-Butyl-5-Choloromethyl-3-Methyl-4-Oxoimidazolidine-1-Carboxylic Acid, T-Butyl Ester, 1.4-Epoxynaphthalene-(2h)-Methanol, 4, 5, 7-Tris (1, 1-Dimethylethyl)-3, 4-Dihydro, etc, have been shown to play a prominent role in protecting the cell wall from degrading agents like salinity, alkalis, acids and even temperature driven stress also (Kampfer, 1994). There are many species which have these adaptations developed in both facultative and obligatory modes, the later ones are restricted to hyper saline environments only but, the former are widespread and can be termed as haloalkaliphilic in nature if they are able to survive the salinity and alkalinity and comprise of *Halomonas*, *Halobacterium, Halovibrio, Bacillus* etc (Arahal and Ventosa, 2002). But *Bacillus* sp, which have adapted to thrive in halophilic environments have much wider applications owing to the wide range of distribution ecologically and their ability to further tolerate /and thrive in multiple restricting factors make them potential organisms to the current study.

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The haloalkaliphilic *Bacillus* sp., also have multiple applications including, enzyme production, waste degradation (both organic like agricultural and house hold and inorganic from industries like dyes, effluents, etc), metal bioremediation (*via* biosorption), and agricultural applications including plant protection and growth promotion and are easily grown on a variety of substrates with relative ease (Syed, 2016).

The ability of these fascinating organisms has driven many researchers to pursue them in order to gain insight into the molecular basis of their unique properties and ultimately employ those for human purposes like salt water agriculture, desalination of salt water, treatment of acidic or alkaline effluents from industries (leather units, paper mills, textiles, etc), reforestation of contaminated lands to prevent desertification (Ghozlan *et al.,* 2006; Syed, 2016).

The current study employs three isolates of haloalkaliphilic *Bacillus* sp. which showed considerable tolerance to temperature (45°C for optimal growth) and alkalinity (pH 12 for optimal growth) along with moderate salinity (15% salinity threshhold) namely *B. licheniformis NSPA5, B. subtilis NSPA8* and *B. cereus NSPA13* isolated from solar salterns of Nellore district (Syed and Paramageetham, 2015).

MATERIALS AND METHODS

Fatty acid methyl ester (FAME) analysis

The fatty acid methyl ester analysis was carried out by initial growth on standard media; Trypticase soy broth agar medium (TSBA), extraction of cellular fatty acids as their methyl esters followed by GC-MS analysis and finally comparing the GC-MS data with instant libraries The extraction processes involved as follows.

Approximately 20 mg (wet weight) of cells were harvested from the streaked plates and transferred into culture tubes for whole cell fatty acid extraction. The cells were saponified with saponification reagent (The saponification reagent consists of 45 gm of sodium hydroxide in 150 ml of methanol and 150 ml of distilled water). Then culture tubes were capped, vortexed for 10 sec, incubated at 100°C for 5 min and vortexed and again incubated for 25 min and cooled to room temperature. The saponification step lyses the cells and converts the released fatty acids into their sodium salts. These fatty acids were methylated with 2.0 ml of methylation reagent (The methylation reagent consists of 325 ml of 6.0 N HCl and 275 ml methyl alcohol). The tubes were vortexed for 5 sec and placed in water bath at 80° C for 10 min and cooled to room temperature immediately to minimise degradation of fatty acid methyl esters. Then the poorly soluble FAMEs were extracted using 1.25 ml of 1:1 (v/v) solution of hexane and methyl-tert-butyl ether. After capping, the culture tubes were mixed end-over-end continuously for 10 min on a laboratory mixer and the aqueous (lower) phase was extracted using a sterile glass Pasteur pipette. For sample clean up, 3.0 ml of sample clean up reagent was used (The sample clean up reagent consists of 0.27 N NaOH in distilled water). A 3.0 ml of clean up reagent was added to the tubes, rotated again continuously for 5 min. Two thirds of the organic (upper) phase was transferred with glass Pasteur pipette into a glass gas chromatography vial, which was sealed with Teflon-lined caps (Sasser, 1990).

FAMEs were quantified using a Perkin Elmer Clarus 680 Gas Chromatography-Clarus 600 Mass Spectrometer equipped with flame ionization detector and ultra 2-Capillary column. The stationary phase of this column was cross linked to the silica tube which provides low noise and drifting during temperature programmed runs. The temperature program was from 170° C to 270°C for 2 min to clean the column off extraneous material and decrease the possibility of carry over. The inlet temperature was 250° C and it was kept constant at a pressure of 9.0 psi. This gave an initial hydrogen carrier flow of approximately 0.4 ml per minute. The flame ionisation detector temperature was 280° C. The makeup gas was ultra high purity nitrogen set at a constant flow rate of 30 ml per minute. Ultra high purity hydrogen and air to the detector were 40 and 350 ml per minute respectively.

The individual FAMEs were identified by comparisons made by using the National Institute of Standards and Technology (NIST) mass spectral El library (NIST 2014/EPA/NIH) with the obtained Electrical Ionisation peaks of the respective fatty acids from the three isolates.

Hydrocarbon tolerance of the Haloalkaliphilic Bacillus sp.

The hydrocarbon tolerance was measured by adding the particular hydrocarbon in to the liquid medium (Nutrient broth) at 5, 10 and 15 per cent followed by inoculating the same with the isolates NSPA5, NSPA8, and NSA13 individually and measuring the optical density

Table 1. Fatty Acid Methyl Esters analysis of *B. licheniformis* NSPA5

S. No	RT	Name of the Compound	Abundance %	Formula	M.W	CAS NO
1	2.533	Heptane, 1, 1'-Oxybis-[2]	3.314	C14H30O	214	629-64-1
2	2.608	1-Phenyl-5-Methylheptane	4.444	C14H22	190	103240-92-2
3	2.879	Propionic Acid, 2, 2-Dimethyl-, 2-Ethylhexyl Ester	4.230	C13H26O2	214	16387-18-1
4	3.694	O-Oxylene [2]	2.178	C8H10	106	95-47-6
5	4.604	1, 2, 4, 5-Tetrazine, 1, 4-Diethylhexahydro-	1.368	C6H16N4	144	35035-69-9
6	5.795	Dodecane, 1-Fluoro-[6]	7.404	C12H25F	188	334-68-9
7	8.586	Benzene, 1, 1'-(1, 1, 2, 2-Tetramethyl-1, 2-Ethanediyl) Bis-	7.151	C18H22	238	1889-67-4
8	8.966	Hentriacontane [6]	12.714	C31H64	436	630-04-6
9	17.159	1, 2-Benzenedicarboxylic Acid, Bis (2-Methylpropyl) Ester	2.338	C16H22O4	278	84-69-5
10	17.645	7, 9-Di-Tert-Butyl-1-Oxaspiro (4, 5) Deca-6, 9-Diene-2, 8-Dione	3.828	C17H24O3	276	82304-66-3
11	17.790	Tetradecanoic Acid, 10, 13-Dimethyl-, Methyl Ester	4.164	C17H34O2	270	267650-23-7
12	18.130	Dibutyl Phthalate	2.550	C16H24O4	278	84-74-2
13	23.132	1, 2-Benzenedicarboxylic Acid, Mono (2-Ethylhexyl) Ester	4.750	C16H22O4	278	4376-20-9
14	25.203	2, 6, 10, 14, 18, 22-Tetracoashexaene, 2, 6, 10, 15, 19, 23-Hexamethyl-, (All-E)-[2]	4.626	C30H50	410	111-02-4

after a incubation period of 72 hrs and at constant stirring at 37°C. A separate series with only pure hydrocarbon was maintained to assay the hydrocarbon degradation potential of the isolates with similar treatment (Margesin and Schinner, 2001).

Heavy metal tolerance of the haloalkaliphilic Bacillus sp.

The heavy metal tolerance was assayed by using diffusion method on nutrient agar medium (NAM); the solid media was inoculated with the isolates via spread plate technique and the metal solutions prepared as their solutions with 1000 ppm final concentration were placed onto 3 mm discs and a volume of 50 µl was added sequentially to make up to 200 µl and incubated for 24 hrs at 37°C, and the zone of inhibition around each disc was measured in mm (Hassen *et al.,* 1998).

RESULTS AND DISCUSSION

Fatty acid methyl ester (FAME) analysis

The fatty acid composition varied greatly among the three isolates *B. licheniformis NSPA5, B. subtilis NSPA8* and *B.cereus NSPA13.* Different fatty acid variations in their proportions gave rise difference in their response to the assays further carried out. Primarily, the fatty acid composition was assayed using GC-MS assisted FAME analysis of the three isolates *B. licheniformis NSPA5, B. subtilis NSPA8* and *B. cereus NSPA13* and the obtained chromatograms are given in Fig. 1, 2, and 3. The chromatogram clearly indicates the variations as well as the change in the intensity of the peaks also, which might lead to the variations of further analysis. The identified peaks from the NIST library are provided in table-1, 2, and 3. The fatty acids like Heptane, 1, 1'-Oxybis-, Dodecane, 1-Fluoro-, Benzene, 1, 1'-(1, 1, 2, 2-

Table 2. Fatty Acid Methyl Esters analysis of *B.subtilis* NSPA8

S. No.	RT	Name of the Compound	Abundance %	Formula	M.W	CAS-NO
1	2.538	Heptane, 1, 1'-Oxybis	2.678	C14H30O	214	629-64-1
2	2.608	1-Phenyl-5-Methylheptane	3.801	C14H22	190	103240-92-2
3	3.819	O-Xylene	1.883	C8H10	106	95-47-6
4	4.314	Thiazole	1.478	C3H3NS	85	288-47-1
5	4.599	1, 24, 5-Tetrazine, 1, 4-Diethylhexahydro-	1.964	C6H16N4	144	35035-69-9
6	8.576	Benzene, 1, 1'-(1, 1, 2, 2-Tetramethyl-1, 2-Ethanediyl) Bis-	5.768	C18H22	238	1889-67-4
7	8.956	Hentriacontane [7]	23.947	C31H64	436	630-04-6
8	10.407	Dodecane, 1-Fluoro- [2]	3.270	C12H25F	188	334-68-9
9	17.144	1, 2-Benzenedicarboxylic Acid, Bis (2-Methylpropyl) Ester	2.151	C16H22O4	278	84-69-5
10	17.619	7, 9-Di-Ert-Butyl-1-Oxaspiro (4, 5) Deca-6, 9-Diene-2, 8-Dione	3.325	C17H24O3	276	82304-66-3
11	17.764	Tetradecanoic Acid, 10, 13-Ddimethyl-, Methyl Ester	3.120	C17H34O2	270	267650-23-7
12	18.105	Dibutyl Phthalate	2.023	C16H22O4	278	84-74-2
13	23.112	1, 2-Benzenedicarboxylic Acid, Mono (2-Ethylhexyl) Ester	3.899	C16H22O4	278	4376-20-9
14	24.012	1.4-Epoxynaphthalene-(2h)-Methanol, 4, 5, 7-Tris(1, 1-Dimethylethyl)-3, 4-Dihydro	31.079	C23H36O2	344	56771-86-9
15	30.374	2-T-Butyl-5-Cholоromethyl-3-Methyl-4-Oxoimidazolidine-1-Carboxylic Acid, T-Butyl Ester	16.252	C14H25O3N2CL	304	900192-88-5

Tetramethyl-1, 2-Ethanediyl) Bis-, Hentriacontane, 1, 2-Benzenedicarboxylic Acid, Bis (2-Methylpropyl) Ester, 7, 9-Di-Tert-Butyl-1-Oxaspiro (4, 5) Deca-6, 9-Diene-2, 8-Dione, Tetradecanoic Acid, 10, 13-Ddimethyl-Methyl Ester, 1, 2-Benzenedicarboxylic Acid, Mono (2-Ethylhexyl) Ester were present in all the isolates, whereas N-(4-Methylbenzenesulfonyl)-2-Methylazetidin-3-One, Propionic Acid, 2, 2-Dimethyl-, 2-Ethylhexyl Ester, Melonic Acid, Decyl Isobutyl Ester, Phenol, 3, 5-Bis(1, 1-Dimethylethyl)-, were present in only isolate *B. cereus NSPA13,* and *B. licheniformis NSPA5, B. subtilis NSPA8* showed over all similar fatty acid composition as evident from table-4 (Toshi, 1968; Welch, 1991).

Hydrocarbon tolerance of the Haloalkaliphilic Bacillus sp.

The hydrocarbon tolerance assay showed all the three isolates *B. licheniformis NSPA5, B. subtilis NSPA8* and *B. cereus NSPA13* were unable to degrade all the tested hydrocarbons (petrol, diesel, xylene, toluene, and kerosene) but showed tolerance to their presence in the medium up to 15 per cent in case of petrol, diesel and kerosene, and the same was limited to 10 per cent in case of xylene and toluene. Among the *B. licheniformis NSPA5, B. subtilis NSPA8* and *B. cereus NSPA13* isolates *B. cereus NSPA13* showed maximum tolerance of hydrocarbons.

Heavy metal tolerance of the haloalkaliphilic Bacillus sp.

The metal tolerance was also diverse in the three isolates *B. licheniformis NSPA5, B. subtilis NSPA8* and *B. cereus NSPA13.* The isolate *B. subtilis NSPA8* is the least tolerant among the three assayed, showing the sensitivity towards six of the metals tested followed by *B.cereus NSPA13* with sensitivity towards five metals. The most tolerant to the tested metals was isolate *B. licheniformis NSPA5* with sensitivity to only three metals. But, as common trait all were sensitive to mercury, lead,

Table 3. Fatty Acid Methyl Esters analysis of *B. cereus* NSPA13

S. No	RT	Name of the Compound	Abundance %	Formula	M.W	CAS-NO
1	2.538	Heptane, 1, 1'-Oxybis-	1.931	C14H30O	214	629-64-1
2	2.608	N-(4-Methylbenzenesulfonyl)-2-Methylazetidin-3-One	2.360	C11H13O3NS	239	76543-28-7
3	2.879	Dodecane, 1-Fluoro- [4]	8.928	C12H25F	188	334-68-9
4	8.576	Benzene, 1, 1'-(1, 1, 2, 2-Tetramethyl-1, 2-Ethanediyl) Bis-	4.386	C18H22	238	1889-67-4
5	8.961	Melonic Acid, Decyl Isobutyl Ester	2.555	C17H32O4	300	900349-10-6
6	11.772	Hentriacontane [8]	9.80	C31H64	436	630-04-6
7	12.753	Trans-2-Methyl-4-N-Pentylthiane, S, S-Dioxide	5.628	C11H22O2S	218	900215-75-3
8	15.169	Chloroacetic Acid, Tetradecylester	3.280	C16H31O2CL	290	18277-86-6
9	17.144	1, 2-Benzenedicarboxylic Acid, Bis (2-Methylpropyl) Ester	1.687	C16H22O4	278	84-69-5
10	17.620	7, 9-Di-Tert-Butyl-1-Oxaspiro(4, 5) Deca-6, 9-Diene-2, 8-Dione	2.692	C17H24O3	276	82304-66-3
11	17.765	Tetradecanoic Acid, 10, 13-Ddimethyl-, Methyl Ester	3.043	C17H34O2	270	267650-23-7
12	23.102	1, 2-Benzenedicarboxylic Acid, Mono (2-Ethylhexyl) Ester	3.437	C16H22O4	278	4376-20-9
13	23.987	Phenol, 3, 5-Bis (1, 1-Dimethylethyl)-	23.412	C14H22O	206	1138-52-9
14	30.350	2-T-Butyl-5-Cholоromethyl-3-Methyl-4-Oxoimidazolidine-1-Carboxylic Acid, T-Butyl Ester	16.435	C14H25O3N2CL	304	900192-88-5

and cadmium. The metal tolerance patterns of the isolates were different from hydrocarbon tolerance, clearly an indication of the dependence of the cellular fatty acid composition on the stress tolerance of the haloalkaliphilic *Bacillus* sp (Nieto *et al.,* 1989; Margesin and Schinner, 2001).

CONCLUSION

The fatty acid composition and variance in the overall fatty acid composition both in type and content showed great difference in the stress resistance towards the limiting factors - different hydrocarbons and heavy metals. The results revealed the cell wall fatty acids play a crucial role in the microorganisms living in harsh and extreme conditions. Metals like copper, chromium received variable response from the isolates, might be indicative of variance in the interaction of cell wall molecules and the metal ions. In order to understand the molecular basis of this kind of variance and the ability to resist the harsh conditions, further studies are required with comparative emphasis on extreme environments.

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Fig. 1. Gas Chromatography-Mass Spectroscopy Spectrum of Fatty Acid Methyl Esters of *B. lichineformis* NSPA5

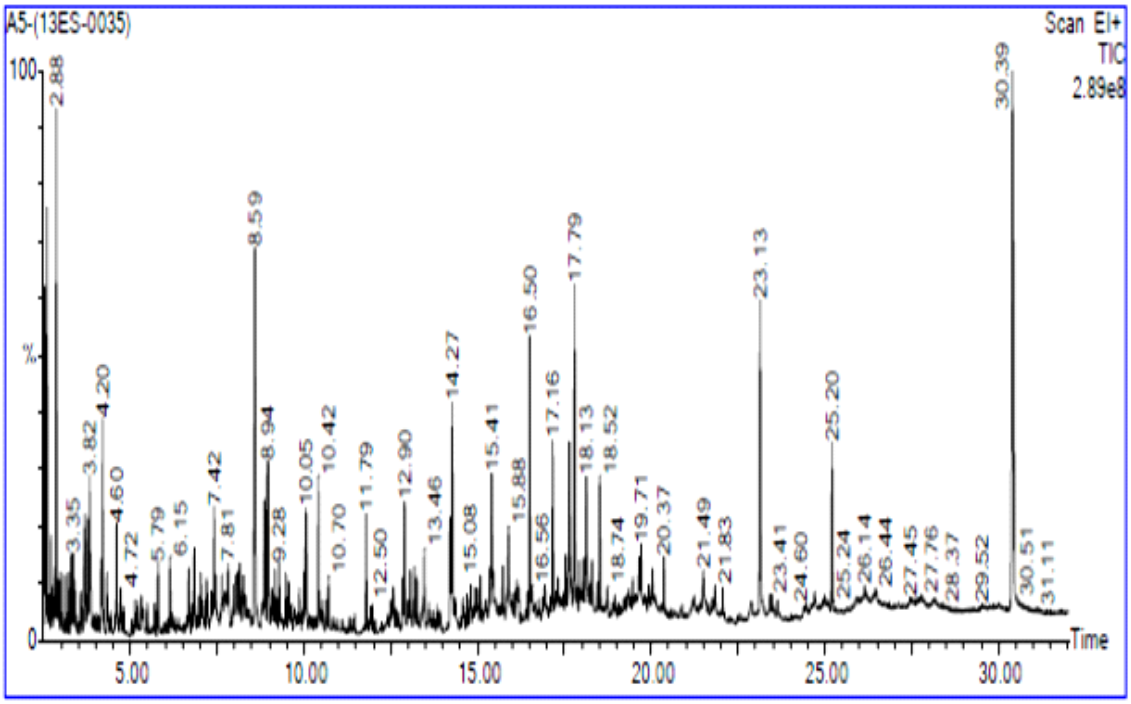


Fig. 2. Gas Chromatography-Mass Spectroscopy Spectrum of Fatty Acid Methyl Esters of *B. subtilus* NSPA8

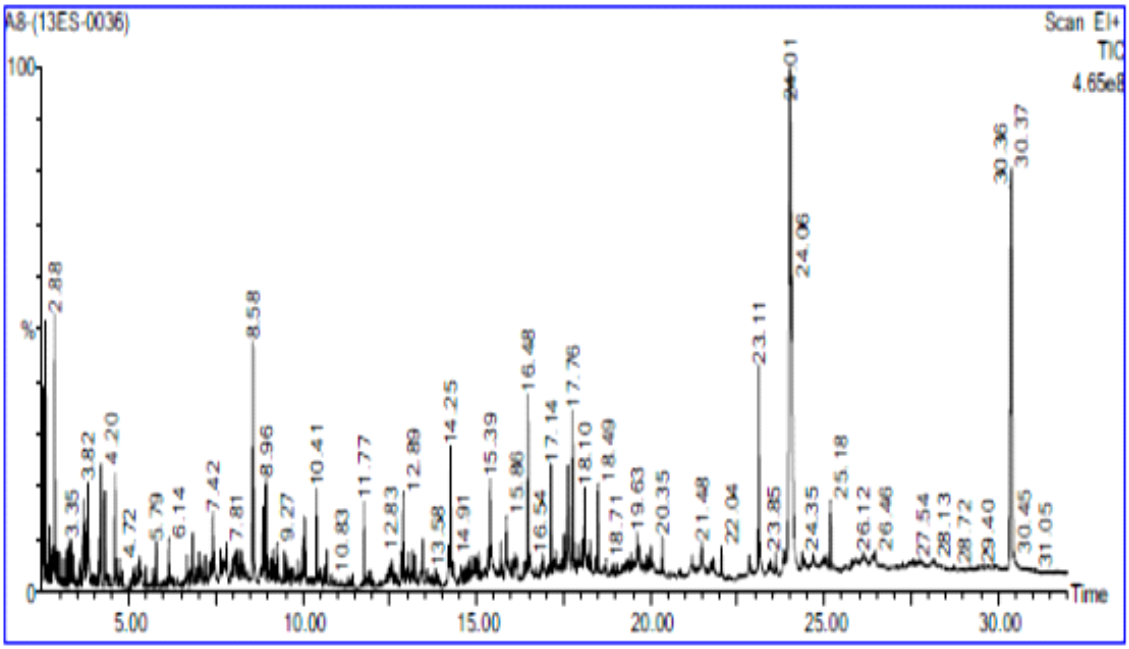
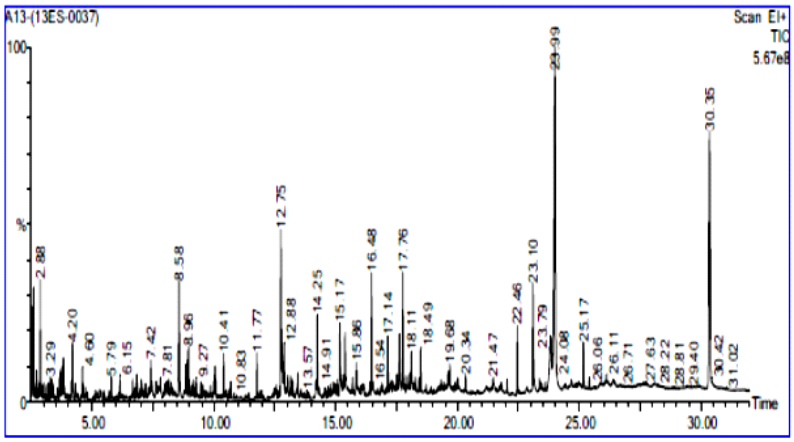


Table 4. Fatty Acid Methyl Ester profiles of the *B. licheniformis* NSPA5 *B. subtilis* NSPA8 and *B. cereus* NSPA13

S. No	RT	Name of the Compound	Abundance %		
			NSPA5	NSPA8	NSPA13
1	2.533	Heptane, 1, 1'-Oxybis-	3.314	2.678	1.931
2	2.608	1-Phenyl-5-Methylheptane	4.444	3.801	-
3	2.608	N-(4-Methylbenzenesulfonyl)-2-Methylazetidin-3-One	-	-	2.360
4	2.879	Propionic Acid, 2, 2-Dimethyl-, 2-Ethylhexyl Ester	-	-	2.360
5	2.879	Dodecane, 1-Fluoro-	7.404	3.270	8.928
6	3.694	O-Oxylene	2.178	1.883	-
7	4.314	Thiazole	1.478	-	-
8	4.599	1, 2, 4, 5-Tetrazine, 1, 4-Diethylhexahydro-	1.368	1.368	-
9	8.576	Benzene, 1, 1'-(1, 1, 2, 2-Tetramethyl-1, 2-Ethanediyl)Bis-	7.151	5.768	4.386
10	8.961	Melonic Acid, Decyl Isobutyl Ester	-	-	2.555
11	8.956	Hentriacontane	12.714	23.947	9.80
12	12.753	Trans-2-Methyl-4-N-Pentylthiane, S, S-Dioxide	-	-	5.628
13	15.169	Chloroacetic Acid, Tetradecylester	-	-	3.280
14	17.144	1, 2-Benzenedicarboxylic Acid, Bis(2-Methylpropyl)Ester	2.338	2.151	1.687
15	17.620	7, 9-Di-Tert-Butyl-1-Oxaspiro (4, 5)Deca-6, 9-Diene-2, 8-Dione	3.828	3.325	2.692
16	17.765	Tetradecanoic Acid, 10, 13-Ddimethyl-, Methyl Ester	4.164	3.120	3.043
17	18.105	Dibutyl Phthalate	2.550	2.023	-
18	23.112	1, 2-Benzenedicarboxylic Acid, Mono(2-Ethylhexyl)Ester	4.750	3.899	3.437
19	23.987	Phenol, 3, 5-Bis(1, 1-Dimethylethyl)-	-	-	23.412
20	24.012	1.4-Epoxynaphthalene-(2h)-Methanol, 4, 5, 7-Tris(1,1-Dimethylethyl)-3, 4-Dihydro	-	31.079	-
21	25.203	2, 6, 10, 14, 18, 22-Tetracoashexaene, 2, 6, 10, 15, 19, 23-Hexamethyl-, (All-E)-[2]	4.626	-	-
22	30.374	2-T-Butyl-5-Cholоromethyl-3-Methyl-4-Oxoimidazolidine-1-Carboxylic Acid, T-Butyl Ester		16.252	16.435

Fig. 3. Gas Chromatography-Mass Spectroscopy Spectrum of Fatty Acid Methyl Esters of *B.cereus* NSPA13



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