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Screening of Marine Fatty Acids Producing Bacteria

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Abstract: Thirty eight bacterial isolates were isolated from sediment samples during summer 2014. Screening of fatty acids production by using H_2O_2-plate assay method and TTC colorimetric method were performed. From few selected false positive strains all strains were found to give remarkable response for all concentration of H_2O_2 and high result for TTC when their lipid extract was injected into GC. The most promising marine bacterial isolate in the production of fatty acids was submitted to the phenotypic characterization through morphological, physiological, biochemical tests and genotypic characterization through 16S rDNA technique and was identified as *Bacillus toyonensis* strain GAD1. The Fatty acids profile of *Bacillus toyonensis* strain GAD1 produced after different times of incubation showed that the highest production of fatty acids was produced after 9 hrs (69.1% of total ex). Fatty acids produced by *Bacillus toyonensis* strain GAD1 showed antibacterial and antifungal activity towards *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterococcus faecalis*, *Salmonella typhi*, *Vibrio* Sp., *Bacillus subtilis*, *Pseudomonas aeruginosa* and for fungal ones *Aspergillus niger*, *Aspergillus flavus* ,*Aspergillus terreus* and *Candida albicans*.
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Introduction

Fatty acids are organic acids characterized by the presence of a carboxyl group (-COOH) at one end and a methyl group (-CH3) at the other end [1]. Fatty acids vary in length and degree of saturation, with naturally occurring fatty acids having a chain length of 4 to 28 carbons which may be saturated or unsaturated [2]. Saturated fatty acids are straight chains and consist of a carbon chain with single bonds, while unsaturated fatty acids contain one or more double carbon-carbon bonds (C=C) which introduces fixed bends into the carbon chain.

Fatty acids are known to possess antibacterial, antimalarial and antifungal activity. The development of resistance of microbes, including fungi and yeasts, towards antimicrobial agents already in use, necessitates the search for alternative antimicrobials, including fatty acids and their derivatives (e.g. methylated and hydroxyl fatty acids). Although fatty acids may not be as effective as chemical fungicides, they pose less environmental risks. They are not only biodegradable, but exhibit a high degree of specificity. In addition, fatty acids are accepted food additives and importantly, pathogenic fungi are less likely to become resistant to antifungal fatty acids. The most important target of antifungal fatty acids is the cell membrane. They cause an increase in membrane fluidity, which will result in leakage of the intracellular components and cell death. Other targets include protein synthesis, which may be inhibited by myristic acid analogues, fatty acid metabolism as well as topoisomerase activity which may be inhibited by amongst others acetylenic fatty acids [3].

Recently the microbial production of poly unsaturated fatty acids (PUFAs) has gained more attention and a number of companies started to produce them commercially and introduce them to the market. Microorganisms producing PUFAs through fermentation provide a good and renewable source of PUFAs as the high omega-3 levels in fish are due to the intake of marine microorganisms since the microorganisms are the only de-novo source of PUFAs.

The main sources of PUFAs in the microbial world are marine algae, fungi and bacteria. Some PUFAs protects bacterial cells not only against the extreme cold environments but also against the high pressure stress [4]**.** In addition to its role in low temperature adaptation, some PUFAs, were found to have a protective role as an antioxidant and may increase the resistance of bacterial cells, to such harmful compounds, by enhancing the biosynthesis of proteins such as porins and TolC family proteins which are involved in the efflux of these harmful compounds [5],[6]**.** Also, the presence, the type and the absence of PUFAs inside the bacterial cell have been used for reclassification and chemotaxonomy for a number of bacterial species [7]**.**

Materials and Methods

Isolation of marine bacteria

All pure isolates used in this study were isolated from marine water samples of Egyptian Red Sea coastline and Alexandria, Egypt. Colonies were collected according to the morphological variations and sub-cultured on NA medium slants at 4ºC in refrigerator for further studies.

Screening of Fatty acids production

Thirty eight marine bacterial isolates were tested for their ability to produce fatty acids using 1) H_2O_2 reaction which nutrient broth medium was used for seed culture preparation and cultivation of selected marine isolates in shake flasks at 180 rpm, 30°C for 24 h. An inoculum was taken from previously prepared seed cultures (OD_{660} ~ 1) and used to inoculate nutrient agar medium containing plate. Different concentration of H_2O_2 (0. 1, 0.5, 1.0% prepared from 30% stock solution) filter paper discs of diameter 5 mm were added on the plates surface and incubated at 30°C for 24 h. Zone of inhibition was observed and further confirmation of FA production performed using GC [8]. Colorimetric screening which 0.1% w/v of the dye 2, 3, 5-triphenyltetrazolium chloride (TTC) was added to 10 ml of the thirty eight bacterial isolates broth. Tubes were incubated at 25°C for one hour. The formation of red color was considered to be a positive result [9]**.**

Quantification of Fatty acids production

The quantity of the produced fatty acids was measured using two different methods; **Spectrophotometer method:** The same procedure mentioned in the colorimetric screening was employed followed by spectrophotometric measurements at 485λ. **Gas chromatography method:** Samples containing fatty acids were prepared according to fatty acid methyl ester preparation method (FAME) [10]**,** [11]. Measurements were employed for both cells and supernatants of selected cultures by harvesting 50 ml of each one. Samples processed by saponification**,** methylation, and extraction and finally subjected to gas chromatograph device model: HP (Hewlett Packard) 6890 GC.

Characterization of the selected marine bacteria

Phenotypic characterization

Phenotypic characteristics such as Gram staining, motility, cultural characteristics, catalase, oxidase, and IMViC test were determined for all the marine bacterial isolates according to the standard methods. Effect of sodium chloride, pH level, and temperature on growth was tested as well. Cell morphology was microscopically examined using electron microscope model JEOL-JSM 5300 at electron microscope unit of central laboratory of NIOF.

Genotypic characterization

DNA of the promising bacterial isolate was isolated and purified with genomic DNA extraction protocol of Gene JET™ genomic DNA purification Kit (Fermentas). The region of 16S rDNA was amplified using Maxima Hot Start PCR Master Mix (Fermentas) and cleaned up using Gene JET™ PCR Purification Kit (fermentas) following the protocol of Sigma Scientific Services Co. Egypt. The sequencing of PCR product was done by GATC Company using ABI 3730xl DNA sequencer by using universal primers for 16S DNA (27F: AGAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACTT .

Growth curve

NB medium was used for seed culture preparation and production of fatty acids in shake flasks. A standard inoculum (1%) was taken from previously prepared seed cultures (OD_{600} ~ 1.2) and used to inoculate 100 ml portions of NB medium dispensed in 500 ml shake flasks. Samples were taken regularly at time intervals of 3h to measure growth and fatty acids by using GC and TTC [12].

Antimicrobial activities of fatty acid methyl esters

Bacterial indicator strains

The antibacterial activity of produced fatty acids and minimum inhibitory concentration (MIC) were examined against three Gram-positive bacteria (*Bacillus subtilis* ATTC 6633, *Stapphylococcus aureus* ATCC 25923 and *Enterococcus faecalies* ATCC 29212) and five Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella Sp., Klebsiella pneumoniae* ATCC 13883 *and Vibrio* Sp.). Antifungal screening was carried out against four fungal species (*Candida albicans* ATCC

10231, *Aspergillus terrus*, *Aspergillus niger and Aspergillus flavus*) .The tested pathogens were kindly provided by the staff members of The National Institute of Oceanography and Fisheries, Egypt.

Agar diffusion well–method [13], [14]

Bacterial indicator strains were cultured in nutrient broth till mid exponential phase. Inocula were uniformly spread on nutrient agar plates and sterile filter paper discs (Whatman No.1; 6 mm) impregnated with 20µl of different concentrations (10, 5 and 2.5 mg) of FAME extract respectively. Blank disc impregnated with hexane that used for the dissolution of fatty acids was used as negative control. Plates were incubated for 18 h at 30 ºC under aerobic conditions. Zone of inhibition was measured in mm and compared with the standard antibiotic Ciprofloxacin (5 µg/disc) was used as positive control. For antifungal activity, sterile filter paper discs (Whatman No.1; 6 mm) impregnated with 20µl of different concentrations (200 µg/disc, 100 µg/disc and 50 µg/disc) of FAME extract. Zone of inhibition was measured after 72 h of incubation and 48 h for candida at room temperature (30°C). Amphotericin B (100 units/disc) was used as positive control.

RESULTS

Isolation and Screening of marine bacteria producing fatty acids

Based on standard isolation methods, Thirty eight bacterial isolates were obtained from sediment samples collected from Egyptian Red Sea coastline (El-Gona station- NIOF Hurghada station - Marsa alam station - Mangrove Safaga station - Shalatin station) and Alexandria coastline during summer 2014. As shown in table 1, Gram staining and morphological examination of 38 bacterial isolates were examined. Out of the total isolates, 13 Gram negative and 25 Gram positive bacteria were detected. After isolation, the total isolates were screened for fatty acids production.

Bacterial screening for Fatty acids production

Screening using H_2O_2 reaction

According to H_2O_2-plate method, cells that are susceptible to externally-added H_2O_2 cannot grow suitably and thus shows a zone of inhibition that is mainly dependent on added concentration of H_2O_2. The diameter of zone is directly proportional to the concentration of external H_2O_2. A paradoxical situation was observed for bacterial cells producing FAs. As shown in table 1, based on varied concentrations of H_2O_2 (1%, 0.5%, 0.1%), 33 isolates were found to give inhibition zone which means no ability for FAs production while 5 isolates forming no inhibition zone that means the ability for FAs production. The five isolates were selected as a promising candida's for the production of FAs.

Screening using colorimetric assay

The reduction of 2,3,5-triphenyltetrazolium chloride (TTC) from a colourless to a red- triphenyl formazan was a convenient indication of the formation of fatty acids. TTC techniques indicated that all isolates (38) produced FAs except AG6 and AG30 (Table 1).

Table 1: Screening of marine bacterial strains for producing fatty acids using H_2O_2-plate assay method and TTC techniques

Isolate Code	H_2O_2 %			TTC	Result
	0.1	**0.5**	**1**		
AG1	-	-	-		+
AG2	-	-	+		+
AG3	-	-	-		+
AG4	-	-	+		+
AG5	-	-	-		+
AG6	-	-	-		-
AG7	-	-	-		+
AG8	-	+	+		+
AG9	+	+	+		+
AG10	+	+	+		+
AG11	-	+	+		+
AG12	-	-	+		+
AG13	-	+	+		+
AG14	-	+	+		+
AG15	-	+	+		+
AG16	-	-	+		+
AG17	-	-	+		+
AG18	+	+	+		+
AG19	-	-	+		+
AG20	-	+	+		+
AG21	-	+	+		+
AG22	-	+	+		+
AG23	-	+	+		+
AG24	-	+	+		+
AG25	-	+	+		+
AG26	+	+	+		+
AG27	-	-	+		+
AG28	+	+	+		+
AG29	-	-	-		+
AG30	-	-	-		-
AG31	-	+	+		+
AG32	-	-	-		+
AG33	-	-	+		+
AG34	-	-	+		+
AG35	-	-	+		+
AG36	-	-	+		+
AG37	-	-	+		+
AG38	-	-	+		+

+ ve describes the presence of FA and −ve denotes FA non producer.

Quantitative measurement of fatty acids produced

Spectrophotometric method

As clearly observed from figure1, isolate AG26 (2,23 nm) followed by AG10 (2,21 nm) showed the highest value of fatty acids produced using TTC techniques. On Contrary, the lowest value was recorded to AG6, and AG30 (0 nm) isolates. According to the highest content of fatty acids produced, five bacterial isolate were chosen to precede gas chromatography [15].

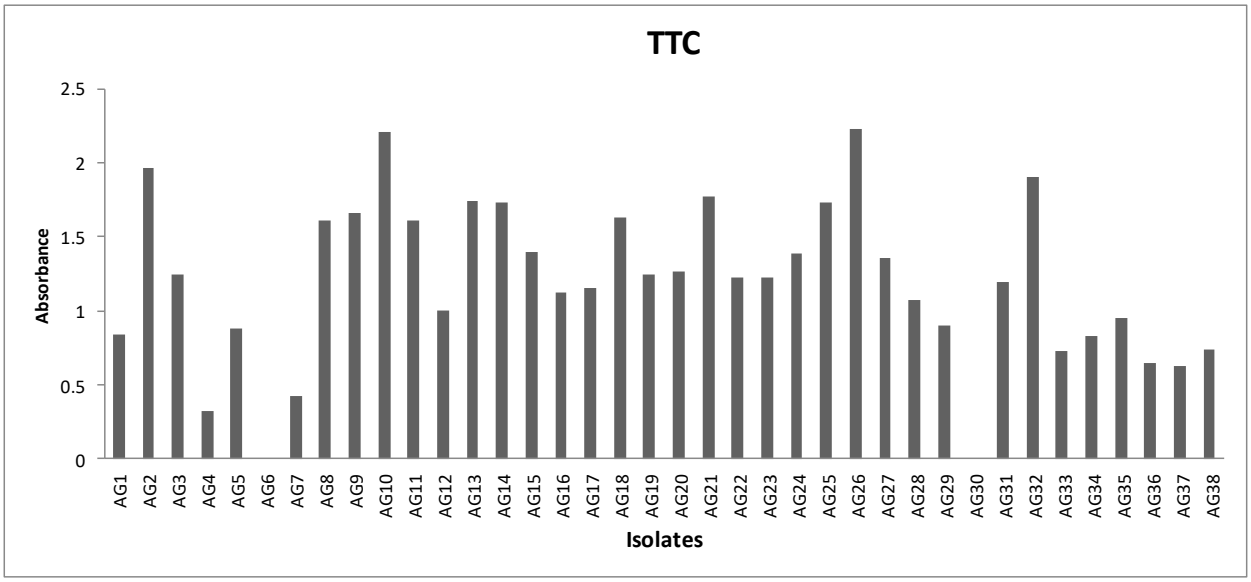

Fig. 1: Production of fatty acids using TTC techniques

Gas chromatography method

Based on both H_2O_2 plate method and the highest values of TTC assay, five isolates were chosen to inject their lipid extract into GC. The spectra of all selected isolates were found to produce different kind of fatty acids. The fatty acid profile of 5 selected isolates was shown in Figure 2 that provides the information about different fatty acids produced by selected marine bacteria after primary screening using H_2O_2-plate assay and TTC.

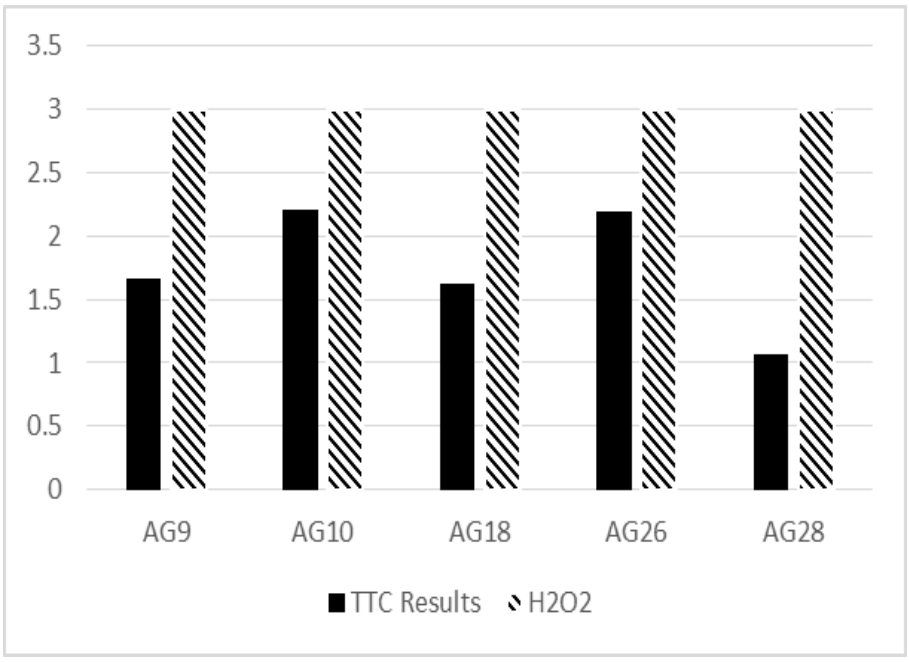

Fig. 2: Selected marine bacteria used for GC screening.

Table 2: Total fatty acids profile for isolate AG9

Fatty acid	FAME	Area of FA	Total area of FA	FA %	Total FA %
C16:0	Palmitic	19.53597	1879	1.0397004	25.0804135
C18:0	Stearic	11.42219		0.6078866	
C22:6	docosahexaenoic	116.3393		6.19155455	
C22:1	Erucic	68.74664		3.65868227	
C22:0	Behenic	255.2168		13.5825896	

Table 3: Total fatty acids profile for isolate AG10

Fatty acid	FAME	Area of FA	Total area of FA	FA %	Total FA %
C16:0	Palmitic	19.241794	1810	1.0630823	29.09429
C18:0	Stearic	26.86716		1.4843735	
C22:2	cis-13,16-Docosadienoic	182.48106		10.0818265	
C22:0	Behenic	91.14613		5.03569779	
C23:0	Tricosanoic	223.15816		12.3291801	

Table 4: Total fatty acids profile for isolate AG26

Fatty acid	FAME	Area of FA	Total area of FA	FA %	Total FA %
C12:0	Lauric	4.951291	2091	0.2367906	30.85239
C13:0	Tridecanoic	7.911124		0.3783417	
C15:1	cis-10-Petadecenoic	6.956299		0.3326781	
C16:0	Palmitic	13.86935		0.6632879	
C18:1	Elaidic, Oleic	7.981		0.3816834	
C20:1	cis-11-Eicosenoic	32.21321		1.5405648	
C22:2	cis-13,16-Docosadienoic Erucic Behenic	201.20514		9.62243615	
C22:1		109.10502		5.21783931	
C22:0		260.931120		12.478771	

Table 5: Total fatty acid profile for isolate AG18

Fatty acid	FAME	Area of FA	Total area of FA	FA %	Total FA %
C18:1	Elaidic, Oleic	2.92711	1476	0.1983137	26.43366601
C18:0	Stearic	11.58335		0.7847798	
C20:0	Arachidic	9.4192		0.6381572	
C22:6	docosahexaenoic	190.84323		12.9297581	
C22:0	Behenic	175.38802		11.8826571	

Table 6: Total fatty acids profile for isolate AG28

Fatty acid	FAME	Area of FA	Total area of FA	FA %	Total FA %
C11:0	Undecanoic	4.61181	2222	0.2075522	16.7863
C14:0	Myristic	4.57671		0.2059725	
C18:1	Elaidic, Oleic	7.61911		0.3428942	
C18:0	Stearic	17.2463		0.7761652	
C22:6	Docosahexaenoic	174.655		7.860268676	
C23:0	Tricosanoic	164.205		7.3902567	

The fatty acids profiles produced using GC for the 5 selected isolates were shown in Figure 3 that provides information about the total fatty acids produced. As clearly observed from this figure, AG26 isolate showed the highest titer for fatty acids production with value of 30.852%.

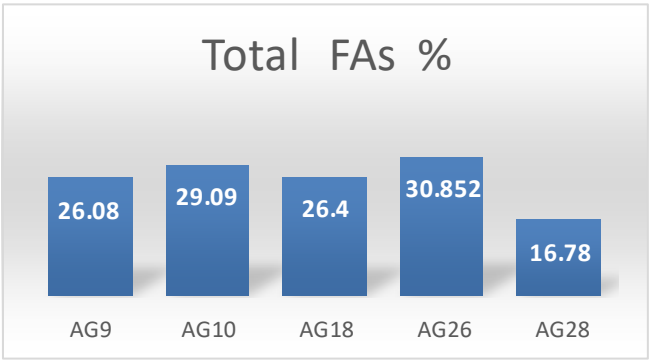

Fig. 3: Showing fatty acids profile using GC of the selected isolates.

Characterization of the selected marine microorganisms

Identification of the most promising marine bacterial isolate

The most promising marine bacterial isolate AG26 for fatty acids production was subjected to some morphological and physiological tests alongside genotypic characterization through 16S rDNA technique.

Phenotypic characterization

Some phenotypic characteristics of the selected isolate AG26 including colony and cell morphology, Gram reaction, catalase and oxidase test in addition to some physiological and biochemical experiments are summarized in table 7. The results showed that it is a Gram-positive bacillus bacterium, motile, facultative anaerobic rods forming central to subterminal spores in non-swollen sporangia. Under oil immersion magnification, Cells occur singly, in pairs and occasionally in short chains or filaments. From plate's examination, colonies are generally large, flat with entire to undulate edges, and often form swarming rings around the colony extending through the plate. Colonies were found to be Yellow in color and grow at temperature range (10-45ºC), pH range (5-9) and sodium chloride concentrations (0-4%).

Table 7: Phenotypic characterization of AG26 isolate

Test	Result
Morphological characterization	
Shape	Rod shape
Colony color	Yellow
Gram reaction	Gram Positive
Physiological characterization	
Temperature	10-45ºC
Optimal growth temperature	30 ºC
pH tolerance range	5 - 9
Salinity tolerance range (%NaCl)	0 - 4 %
Biochemical characterization	
Catalase	+Ve
Oxidase	+Ve
Indole test from tryptophan	-Ve
Methyl red test	+Ve
Voges proskaur test	-Ve
Citrate test	+Ve
H_2S production test	-Ve
Glucose utilization	+Ve

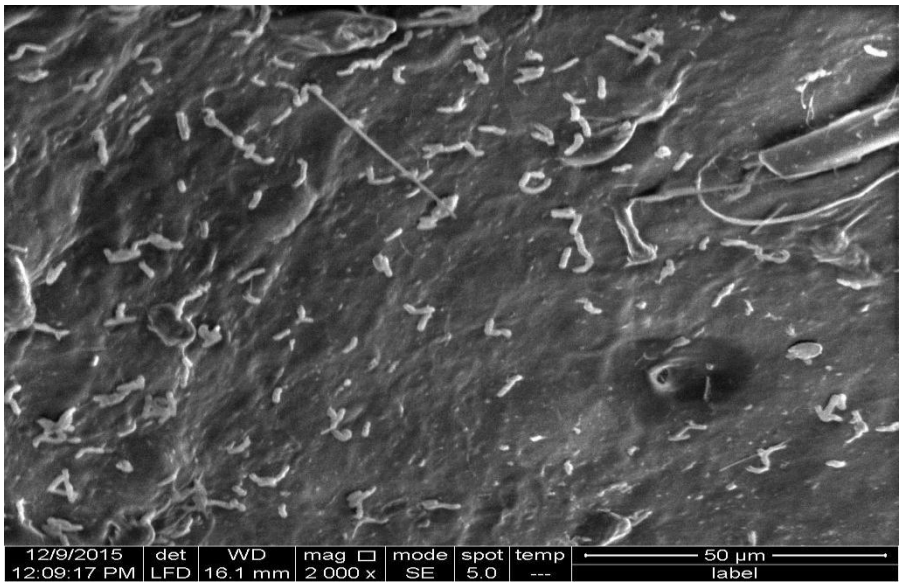

Fig. 4: Scanning electron microscopic feature of bacterial isolate AG26.

Genotypic characterization

The genomic DNA of the bacterial isolate AG26 was prepared, purified and the gene coding for the 16S rDNA was partially amplified using the universal primers (16S 27F and 16S 1492R). The amplified PCR fragment (approximately 1200 bp) was purified and then sequenced Fig. 5. Table 8 shows GenBank accession numbers of 16S rRNA gene partial sequences (719 bp) of the isolate; the highest sequence similarity as well as the closest neighbour(s). Nucleotide sequence of AG26 isolate was affiliated according to its 16S rDNA to members of genus *Bacillus*. For AG26, the first (719bp) showed the highest sequence homology of 100% to *Bacillus toyonensis* Fig. 6.

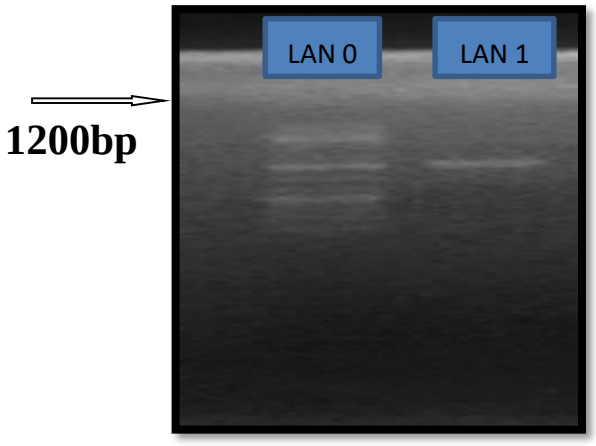

Figure 5: 16S agarose gel electrophoreses of the amplified 16S rDNA gene of the isolate under study. LAN0 (DNA marker 1kpb), LAN1 (16S fragment sample).

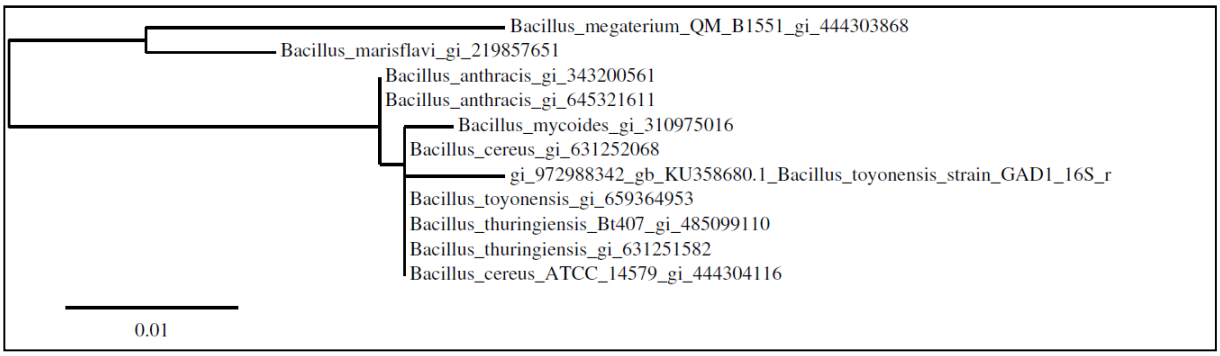

Figure 6: Phylogenetic tree of *Bacillus toyonensis strain GAD1*, 16S rDNA-based dendogram showing the phylogenetic position of isolate AG26 among representatives of related bacterial species.

Table 8: Accession number, similarity % to the nearest neighbors of the tested isolates.

Isolate	Accession no.	Nearest neighbor(s)	Similarity (%)
AG26	**KU358680.1**	***Bacillus toyonensis***	100

***Bacillus toyonensis* strain GAD1 growth curve**

Bacillus toyonensis exhibited a log phase of 12 h and then entered stationary phase. Fatty acid production started after 3h and showed highest titre (69.1 %) after 9 h of growth Fig.7, 8. The increase in fatty acid production was associated with the increase in bacterial growth.

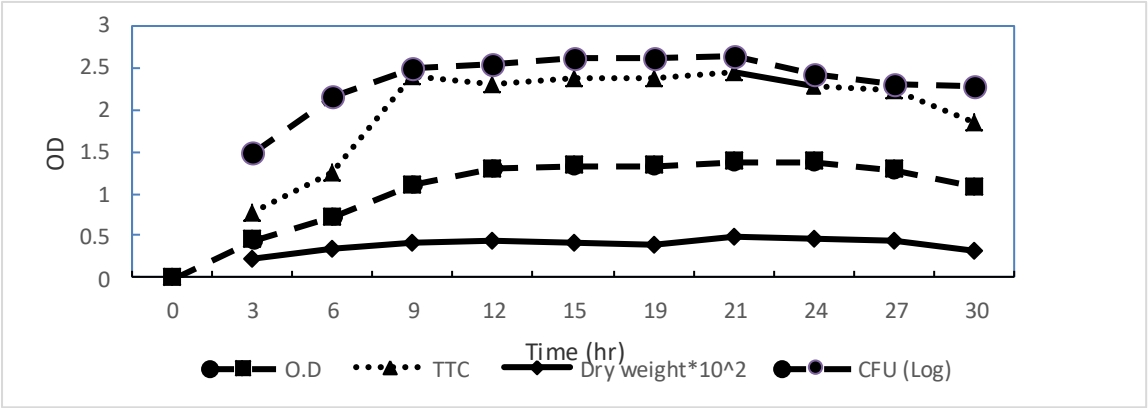

Fig. 7: *Bacillus toyonensis* strain GAD1 bacterial growth curve and fatty acids production by TTC (λ_{485}) methods.

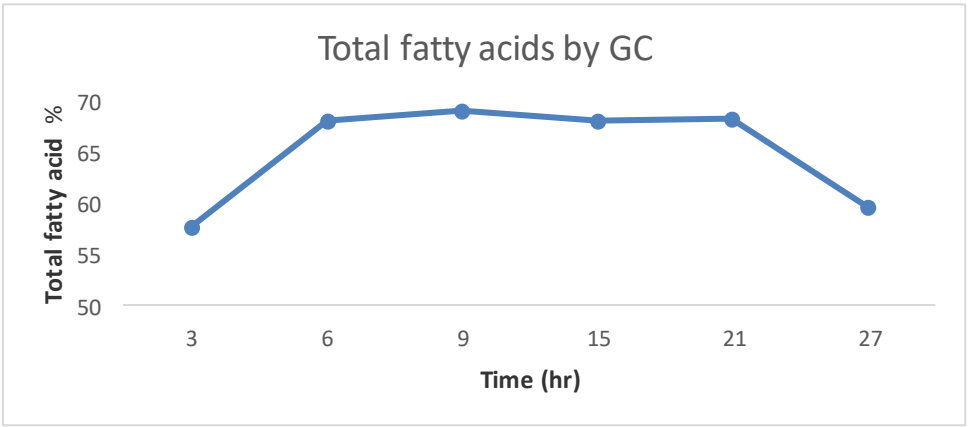

Fig. 8: fatty acids production by GC technique

Table 10: Fatty acids profile of marine isolate *Bacillus toyonensis* strain GAD1 produced after different times of incubation by GC.

Fatty acid	FAME	3 hrs	6 hrs	9 hrs	15 hrs	21 hrs	27 hrs.
C10:0	**Capric**	0.000	0.576	0.000	0.295	1.105	0.000
C11:0	**Undecanoic**	0.000	2.335	2.728	4.585	3.104	1.276
C12:0	**Lauric**	6.791	5.185	4.671	5.687	8.954	2.695
C13:0	**Tridecanoic**	3.087	5.068	7.515	8.025	3.260	3.619
C14:0	**Myristic**	0.000	0.000	0.000	0.000	0.994	0.000
C15:1	**cis-10-Petadecenoic**	4.492	5.880	8.553	8.334	3.883	5.123
C16:0	**Palmitic**	5.568	6.347	9.031	8.573	5.485	6.582
C18:3	**(n-3) α-Linolenic**	6.294	6.174	8.519	7.910	5.407	7.417
C22:6	**docosahexaenoic**	0.000	0.000	0.000	0.000	9.565	0.000
C22:2	**cis-13,16-Docosadienoic**	14.769	16.141	15.086	12.180	13.684	15.106
C22:1	**Erucic**	0.000	7.896	0.000	0.000	0.000	0.000
C22:0	**Behenic**	16.696	12.704	13.009	12.541	12.876	17.866
Total Fatty acid %		**57.697**	**68.308**	**69.113**	**68.129**	**68.318**	**59.684**

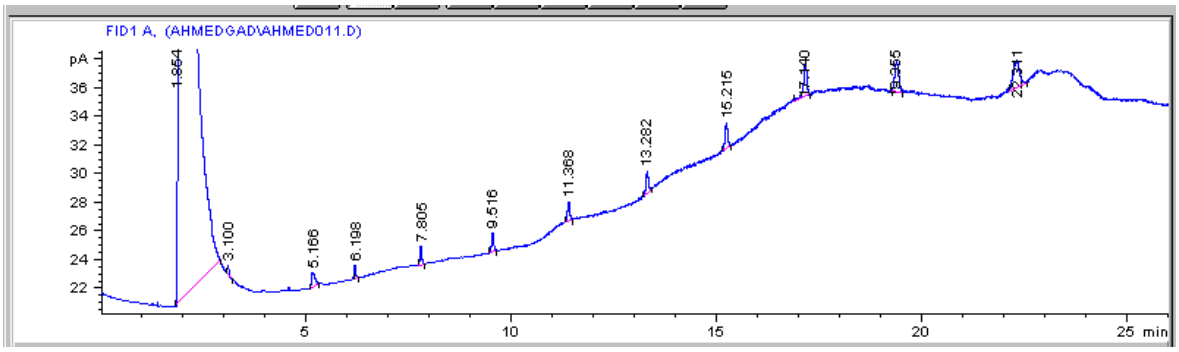

Fig. 8: **Gas chromatogram showing fatty acids profile of a selected marine isolate after 3 hrs of incubation.**

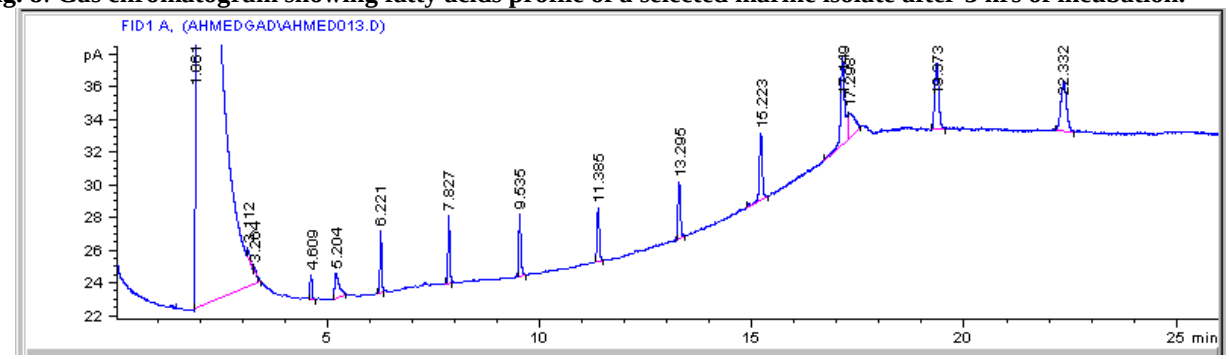

Fig. 9: **Gas chromatogram showing fatty acids profile of a selected marine isolate after 6 hrs of incubation.**

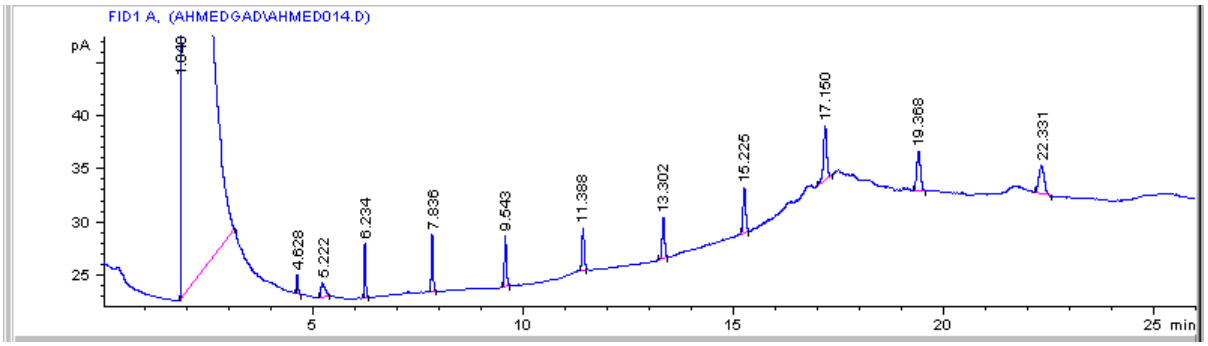

Fig. 10: **Gas chromatogram showing fatty acids profile of a selected marine isolate after 9 hrs of incubation.**

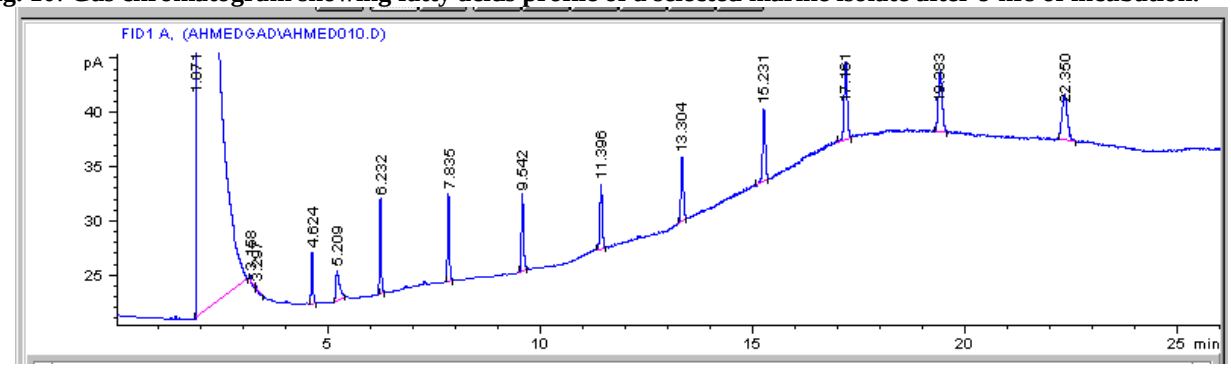

Fig. 11: **Gas chromatogram showing fatty acids profile of a selected marine isolate after 15 hrs of incubation.**

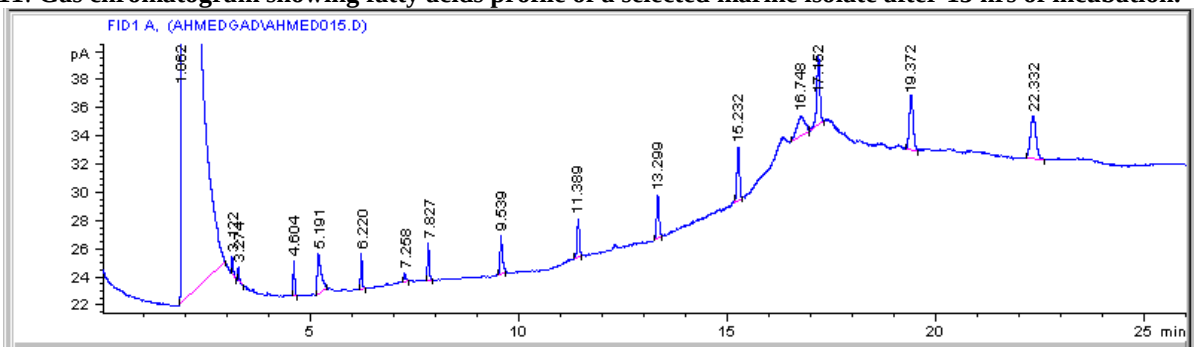

Fig. 12: **Gas chromatogram showing fatty acids profile of a selected marine isolate after 21 hrs of incubation.**

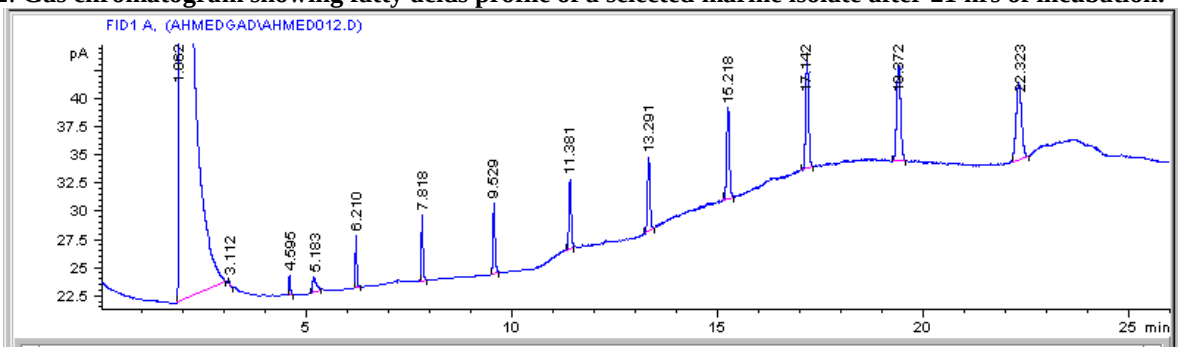

Fig. 13: **Gas chromatogram showing fatty acids profile of a selected marine isolate after 27 hrs of incubation.**

As shown in Table 10, the Fatty acids profile of marine isolate *Bacillus toyonensis* strain GAD1 produced after different times of incubation showed that the highest production of fatty acids was produced after 9 hrs representing about 69.1% of the total extract followed by 21 hrs incubation representing about 68.31%. On the other hand, the lowest fatty acids production was after 3 hrs incubation showing about 57.6% of total extract. The marine bacteria *Bacillus toyonensis* strain GAD1 showed the ability to produce certain fatty acids in specific times of

incubation such as C14:0 and C22:6 which produced only after 21 hr of incubation and C22:1 that produced after 6 hrs of incubation.

Antimicrobial activities of fatty acid methyl esters

Antibacterial Activity:

Data in Fig. 9 reveal that fatty acids produced exhibited antagonistic effect against all tested pathogenic bacteria, with variable degrees depending on bacterial species. The highest activity appeared against *Staphylococcus aureus* followed by *Klebsiella Pneumoniae*, while the lowest activity of fatty acids observed against *Pseudomonas aeruginosa*. These data were recorded after incubation of plates for 24 h at 30 ºC while at 37 ºC; the highest activity of fatty acids was found against *Staphylococcus aureus* followed by *Klebsiella pneumoniae*. The lowest activity observed against *Vibrio* sp. compared to the standard reference Ciprofloxacin (5 µg/disc).

Antifungal Activity:

The antifungal activities of Fatty acids and standard Amphotericin B (100 units/disc) were determined at the concentrations of 50,100 and 200 µg/disc against four pathogenic fungi. The fatty acids extract showed significant activity against all tested pathogenic fungi. The highest activity was observed against *Aspergillus niger and Aspergillus flavus* followed by activity against *Aspergillus terreus* with concentration of 200 µg/disc. The lowest activity was found against *Aspergillus flavus* at the concentration of 50 µg/disc.

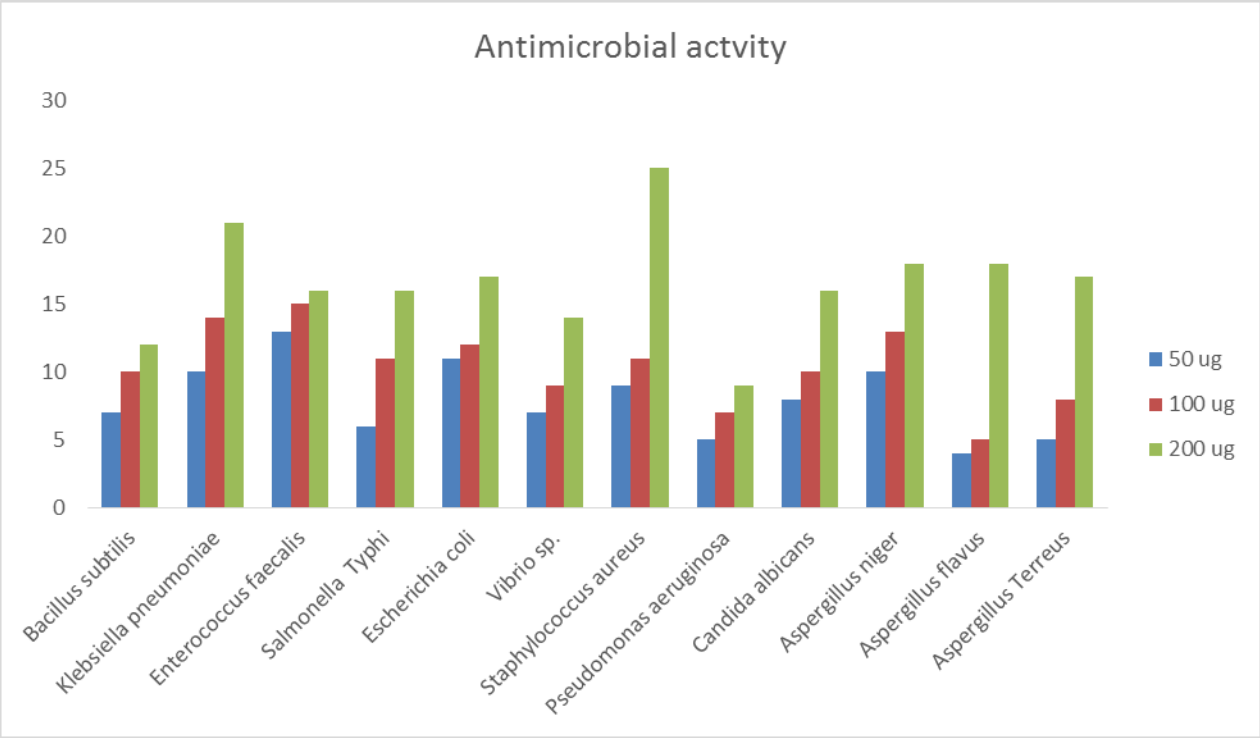

Fig. 9: Antibacterial and antifungal activities of fatty acid methyl esters at 30C, Mean zone of inhibition (mm) at 30 ˚C.

DISCUSSION

Microorganisms producing PUFAs through fermentation provide a good and renewable source of PUFAs as the high omega-3 levels in fish are due to the intake of marine microorganisms since the microorganisms are the only de-novo source of PUFAs. The main sources of PUFAs in the microbial world are marine algae, fungi and bacteria [16].

As a first step, H_2O_2 has been used to quantify the amount of fatty acids produced by marine bacteria, and the total fatty acids contents including the saturated and unsaturated ones have been confirmed by GC analysis. Out of 38 marine bacterial isolates, 5 isolates have shown the ability to produce the fatty acids and were chosen for further screening.

Tilay and Annapure, 2012, [8] have chosen the H_2O_2 assay for selecting PUFA-producing bacteria by direct visualization using the H_2O_2-plate assay as a reliable method for screening of number of strains within a short period of time. This assay has depended on the oxidative stability of PUFAs in growing bacteria towards added H_2O_2 is a distinguishing characteristic between the PUFAs producers (no zone of inhibition) and non-PUFAs producers (zone of inhibition) by direct visualization. EPA- and DHA-expressing bacteria were reported to be more resistant to exogenous H_2O_2 [17].

TTC has been used as a screening test of a bacterial fatty acids production. A direct association between the ability to grow on broth containing TTC, to reduce TTC to TF, and to produce fatty acids have been found [9]**.** In the present study, TCC has been used as a screening tool to quantify a number of fatty acids produced from marine bacteria using spectrophotometer measurements. The obtained results have been confirmed by GC output data. The appearance of changing to red color has indicated a positive result regarding the amount of fatty acids production according to the intensity of the color. This tool for measuring has promoted to be an efficient tool for quantifying the total fatty acids including EPA, DHA, ALA, GLA, and others. Out of 38 bacterial isolates, 5 isolates showed different red color intensity. Those isolates were selected for GC analysis that confirmed the existence of different fatty acids with different amount. [9] have used this approach as a qualitative tool for measuring EPA gaining the advantages of significantly reducing the number of samples submitted for GC analysis and therefore reducing the time, effort and cost involved in the screening and isolation of EPA-producing marine bacteria strains.

Zhu *et al*. (2004), Ryan *et al.* (2010), **Elrazak *et al*. (2013)** [18] [9]**,**[19]**,** have suggested that the enzyme Δ5-desaturase could be the responsible factor for the reduction of the colourless TTC to the bright TF as observed in the production of a different PUFA, arachidonic acid, in the fungal culture of Mortierella alpine.

The selected 5 bacterial isolates (AG9, AG10, AG18, AG26, AG28) were subjected for further screening for the highest production of fatty acids using GC measurements. The isolate AG26 had the highest production of fatty acids was biochemically and genetically identified. The strain was identified as *Bacillus toyonensis* strain GAD1 with 100% sequence homology to *Bacillus toyonensis*.

Bacillus toyonesis is a member of the *B.cereus* family which consists of *B.thuringiensis, B.anthracis, B.mycoides, B.pseudomycoides, B.weihenstephanensis, B.cytotoxicus* and *B.cereus* [20].

Okaiyeto K. *et al.*(2015) [21] observed a similar result when identifying a bacterium isolated from sediment samples of a marine environment in the Eastern Cape Province of South Africa which the isolate showed 100% similarity to *Bacillus toyonensis*.

Bacillus toyonensis strain GAD1 after 3hors of incubation produced 7 different fatty acids representing about 57.69 % of the total extract. The major one (C22:0 Behenic acid) represents about 16.69%. The unsaturated fatty acids represent about (25.54%) and the saturated fatty acids represent about (32.1%) of the total fatty acids content of *Bacillus toyonensis* strain GAD1 after 3hrs of incubation. After 6 hours of incubation, the strain produced 10 different fatty acids representing about 68.3 % of the total extract. The major one (C22:0 Behenic acid) represents about 12.7 %. The unsaturated fatty acids represent about 36.3% and saturated fatty acids represent about 31.9%. After 9 hours of incubation this strain produced 8 different fatty acids representing about 69.1 %. The major one (C22:2 Docosadienoic acid) represents about 15.0 %. The unsaturated fatty acids represent about 32.1% and saturated fatty acids represent about 36.9% of total fatty acids content.

After 15 hours of incubation, 9 different fatty acids representing about 68.1 % of the total extract were produced. The major one (C22:2 Docosadienoic acid) represents about 12.7 %. The unsaturated fatty acids represent about 28.4 % and saturated fatty acids represent about 39.7 % of the total fatty acids content. After 21 hours of incubation produced 11 different fatty acids representing about 68.1 % of total extract. The major one (C22:2 Docosadienoic acid) represents about 16.1 %. The unsaturated fatty acids represent about 36.3 % and saturated fatty acids represent about 31.9 %. After 27 hours of incubation, 8 different fatty acids representing about 59.68 % of the total extract. The major one (C22:0 Behenic acid) represents about 17.86 %. The unsaturated fatty acids represent about 27.6 % and saturated fatty acids represents about 32.0 % of total fatty acids content.

Bacillus toyonesis starin GAD1 showed the ability to produce certain fatty acids in specific times of incubation such as C14:0 and C22:6 which produced only after 21 hrs of incubation and C22:1 that produced only after 6 hrs of incubation .The most likely explanation is that the bacteria produce certain fatty acids in specific times of incubation for adaptation to difficult environmental factors.

Effect of different temperatures on fatty acids production was tested; the experiment showed that 30 ◦C was the optimum one which is lying in the mesophilic temperature zone. Unlikely, [19] indicated that the fatty acids getting more abundant in the cold environment than the temperate environment.

Fatty acids produced by *Bacillus toyonensis* strain GAD1 were examined for antibacterial and antifungal activity. It inhibited *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterococcus faecalis*, *Salmonella Typhi*, *Vibrio Sp.*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and for fungal ones *Aspergillus niger*, *Aspergillus flavus* ,*Aspergillus Terreus* and *Candida albicans*,. These differences could be due to the nature and level of the antimicrobial agents present in the extracts and their mode of action on different test microorganisms [22].

The previous results indicated that Gram-positive bacteria were more susceptible than the Gram-negative bacteria. Similar results were obtained with FAME extracts of leaves of *Ipomoea pes-caprae* and lipophilic extracts of various plant parts of *Pistacia vera* [23], [24].

These differences in the fatty acids sensitivities between Gram-positive and Gram-negative bacteria may result from the impermeability of the outer membrane of Gram-negative bacteria since the outer membrane is an effective barrier against hydrophobic substances [25], [26]. As a fact, Gram negative bacteria are more resistant to inactivation by medium and long chain fatty acids than Gram-positive bacteria, [27], [28], [3] have mentioned that fatty acids and their derivatives hold great potential as environmentally friendly antifungal agents or leads for novel antifungal drugs. The knowledge about the different mechanisms indicated that the cell membrane is a major target for those compounds, but those specific enzymes and metabolic pathways are also targets for these compounds. It is also clear that specific fatty acids (such as certain acetylenic fatty acids) may be active in several pathways.

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