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Production of Polyunsaturated Fatty Acids (PUFAs) From Some Marine Microalgae with Special Emphasis on Eicosapentaenoic Acid (EPA"(

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Abstract: Nine marine microalgae species namely (Pseudanabaena sp, Chlorella sorokiniana, Chlorella salina, Dunaliella salina, Amphora marina and Phaeodactylum tricornutum were selected to test their potentialities for production of polyunsaturated fatty acids (PUFAs). The screening strategy for the production of PUFAs in this study was GC/MS analysis. The results indicated that diatoms followed by green microalgae were the potential producers of PUFAs. Among the tested producers, Phaeodactylum tricornutum found to be the hightest potential alga to produce PUFA relatively high levels of EPA in particular. A Plackett–Burman statistical design of experiments was applied to screen the effect of different factors including Ca(NO3)2.4H2O, K2HPO4.3H2O, MgSO4.7H2O, Na2SiO3.9H2O, Na2CO3, H3BO3, MnCl2.4H2O, CuSO4.5H2O, HMoO4, ZnCl2, CoCl2.6H2O, FeCl3.6H2O, Na2EDTA.2H2O, Spirulina filtrate, Na2SeO3.5H2O, AlCl3 and glycerol as components of a production medium. This optimization strategy led to a significant increase in the amount of EPA produced by Phaeodactylum tricornutum, where the amount of EPA increased from 1.6 mg/g biomass to 14.5 mg/g.

keywords: Diatoms, eicosapentaenoic acid (EPA), Plackett–Burman, Phaeodactylum tricornutum, polyunsaturated fatty acids

1.Introduction

Polyunsaturated fatty acids are those containing two or more double bonds along the carbon skeleton. Most fatty acids found in the body are obtained from the diet. Eicosapentaenoic acid (EPA) is one of the most important FA due to its significant health effect on human. EPA is a type of PUFA with 20 carbon atoms and 5 double bonds so called Long Chain PUFA and it belongs to the omega-3 family as the first double bond is between the third and fourth carbon atoms from the omega end of the carbon atom chain.

The ability of human body to convert alpha linolenic acid (ALA) to eicosapentaenoic acid (EPA) is very limited so that food is the main source of this cis n-3 PUFA in the human body [1].

The health benefits of EPA include decreasing of blood plasma cholesterol and prevention of colon and pancreatic cancers [2]. EPA has a protective effect against atherosclerosis so that the percentage of cardiovascular diseases in human populations with high fish utilization was reported to be significantly low [6, 7]. The ability of EPA to prevent and treat most of the blood-circulatory diseases is due to the antiaggregatory role of EPA and in maintaining homeostasis [8, 9]. Microalgae are known as a good source for PUFAs production because they can synthesize and accumulate large quantities of lipids (20–50% of dry weight of biomass) also they can grow at relatively high rates [3]. Microalgae grow well on non-arable, nutrient-poor land in which plants cannot grow on it [4]. They are promising vegetative and non-polluted sources for LC-PUFA production as an alternative to fish oil. Microalgae are the initial EPA and DHA producers in the marine food chain and can naturally grow fast under a variety of autotrophic, mixotrophic and heterotrophic culture conditions with high long chain ω-3 fatty acid production potential [5].

The primary objective of this research was to screen PUFAs especially EPA production of

different marine microalgae including four diatoms (*Amphora marina* (BIRD BAC402) and *Phaeodactylum tricornutum* (BIRD BAC430, BIRD BAC431, BIRD BAC464)), four green microalgae (*Chlorella sorokiniana* (BIRD CHL120), *Chlorella salina* (BIRD CHL125), *Dunaliella salina*(BIRD CHL136), *Dunaliella salina*(BIRD CHL137)) and one cyanophyte (*Pseudanabaena* sp (BIRD CYN058)).

Owing to the tremendous health benefits of the fatty acid EPA, the most potential producer of this particular PUFA will be selected for further research including series of sophisticated optimization matrices to enhance EPA production.

2 .Materials and methods

Test microalgae strains

Nine marine microalgae isolates including (*Pseudanabaena* sp(BIRD CYN058), *Chlorella sorokiniana*(BIRD CHL120), *Chlorella salina*(BIRD CHL125), *Dunaliella salina*(BIRD CHL136), *Dunaliella salina*(BIRD CHL137), *Amphora marina*(BIRD BAC402) and *Phaeodactylum tricornutum*(BIRD BAC430, BIRD BAC431, BIRD BAC464)) were selected to carry out this study. They were obtained from the culture collection of Biotechnology International Research and Development Centre (BIRD), Mansoura, Egypt.

Growth conditions

All isolates were cultured in 2L flasks containing 1L of modified Navicula nutrient medium as described by Starr [10] after sterilization. An amount of 25 g/L crude sea salt was added to the nutrient medium. Three replicate flasks were inoculated with a single algal species (100 ml) which subsequently incubated in an air-conditioned growth room at 25 ± 2°C with continuous light of 2.789w m^{-2}. The developed biomass of *Pseudanabaena* sp, diatom isolates, *Chlorella sorokiniana*, *Chlorella salina* and *Dunaliella salina* isolates were harvested after 5, 6, 7, 11 and 14 Days of incubation respectively.

Fatty acid methyl ester (FAME) preparation and Gas Chromatography/ Mass Spectroscopy analysis

Hundred mg of freeze-dried cells were suspended in 4 ml of 5% methanolicHCl (5ml HCl + 95ml methanol) and heated at 70°C in a water bath for 2 hours in sealed glass tubes. The tubes were cooled down to room temperature for 30 minutes then 6 ml hexane was added to each glass tubes which were vigorously vortexed to extract FAME. The upper layer containing the extracted FAME was transferred into a clean tube and dried in dessicator. A known equal volume of hexane in which known and equal concentration of the internal standard (nonadecanoic acid C_{19}) was added separately to each tube containing FAME residues of different algal isolates [11].

For the determination of PUFAs concentration, the single point internal standard method was used.The internal standard used was methyl nonadecanoic acid (≥ 99.5% GC capillary purity, Sigma-Fluka). The machine was loaded with silica capillary column PAS-5 ms (30 m × 0.32 mm× 0.25 µm film thickness), and the samples were analyzed using the program described by Laakso *et al*., 2002 [12]. Wiley and Wiley Nist mass spectral data base was used for the identification of the separated peaks. The GC-MS analysis was carried out at Central Agriculture Pesticides Laboratory (CAPL), Dokki, Cairo, Egypt.

Screening the effect of modified Navicula nutrient medium component on biomass and PUFAs production of *Phaeodactylum tricornutum* by Plackett-Burman (PB)

Upon preliminary screening results, *Phaeodactylum tricornutum* was found to be the highest EPA producer (**Table 2**) and as a result; it was selected for further investigations.

A PB experiment design was applied to screen the effect of different nutrients on the growth and productivity of the isolate under investigation. The nutrients to be screened includes $Ca(NO_3)_2.4H_2O$, $K_2HPO_4.3H_2O$,$CoCl_2.6H_2O$, sea salt, $Na_2SeO_3.5H_2O$ [13]

$FeCl_3.6H_2O$, $Na_2EDTA.2H_2O$, H_3BO_3, $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $HMoO_4$, ZnCl $MgSO_4.7H_2O$, $Na_2SiO_3.9H2O$, Na_2CO_3, $_2$,

$AlCl_3$ [14,15], glycerol [16], *Spirulina* filtrate and one dummy factor to evaluate the standard error of the performed design. Each factor was tested at three levels: low (-),

medium (0), and high (+) at concentrations shown in Table 1. The difference between the minimum and maximum values should be neither small, as it may not show the effect, nor large, as it could mask the effect of the others [17].

The validation experiments were carried out to determine the accuracy of the generated models.

Screening for polyunsaturated fatty acids production by the test microalgae

The results of GC/MS analysis revealed that all the tested isolates, except the cyanobacterium *pseudanabaena* sp (BIRD CYN058), are potential producers of a wide variety of PUFAs (**Table 2**).

All the test green microalgae produce ALA with a percentage ranged between

22.97% - 55.63%. In addition to their remarkable production of ALA two green microalgae produce LA in a reasonable

percentage of 38.3% *Chlorella sorokiniana* (BIRD CHL120) and 49.23% *Chlorella salina* (BIRD CHL125). *Dunaliella salina* (BIRD CHL136) also produces ARA with a relative percentage of 4.68%. The results indicated that diatoms are potential producers of all PUFAs assayed but in different relative quantities (% of PUFA/ total fatty acids). The most elegant observation was the relatively higher production of EPA with a percentage ranged between 11.11% (*Phaeodactylum tricornutum* BIRD BAC464) and 14.79% (*Phaeodactylum tricornutum* BIRD BAC431).The diatom *Amphora marina* BIRD BAC402 produced the highest ARA (8.59%) recorded in this study.

The highest EPA production was recorded for the isolates *Phaeodactylum tricornutum* BIRD BAC430 (14.46%) and *Phaeodactylum tricornutum* BIRD BAC431 (14.79). Owing to the fact that EPA maintains potential health benefits for consumers; the isolate *Phaeodactylum tricornutum* BIRD BAC431 was selected for further studies

Table 1: Variables investigated for PUFAs production via Plackett–Burman design for *Phaeodactylum tricornutum*

Variables	Code	Unit	Center point (0)	Minimum level (-)	maximum level (+)
$Ca(NO_3)_2.4H_2O$	X_1	g/L	0.1	0.05	0.15
$K_2HPO_4.3H_2O$	X_2	g/L	0.14	0.07	0.21
$MgSO_4.7H_2O$	X_3	g/L	0.025	0.0125	0.0375
$Na_2SiO_3.9H_2O$	X_4	g/L	0.1	0.05	0.15
Na_2CO_3	X_5	g/L	0.02	0.01	0.03
$FeCl_3.6H_2O$	X_6	g/L	0.005	0.0025	0.0075
$Na_2EDTA.2H_2O$	X_7	g/L	0.03	0.015	0.045
H_3BO_3	X_8	mg/L	2.8	1.4	4.2
$MnCl_2.4H_2O$	X_9	mg/L	0.9	0.45	1.35
$CuSO_4.5H_2O$	X_{10}	mg/L	0.125	0.0625	0.1875
$HMoO_4$	X_{11}	mg/L	0.08	0.04	0.12
$ZnCl_2$	X_{12}	mg/L	0.09	0.045	0.135
$CoCl_2.6H_2O$	X_{13}	mg/L	0.041	0.0205	0.0615
Sea salt	X_{14}	g/L	25	10	40
$Na_2SeO_3.5H_2O$	X_{15}	mg/L	0.85	0.425	1.275
$AlCl_3$	X_{16}	µg/L	25	0	50
Glycerol	X_{17}	M	0.1	0.005	0.2
Spirulina filtrate	X_{18}	ml/L	6	3	9
Dummy	X_{19}	_	0	-1	1

Table 2: Polyunsaturated fatty acid profile for the test isolates (% of total fatty acids)

Isolate	LA%	DGLA%	ARA%	ALA%	EPA%	DHA%
BIRD CYN058	-	-	-	-	-	-
BIRD CHL120	38.3	-	-	26.09	-	-
BIRD CHL125	49.23	-	-	22.97	-	-
BIRD CHL136	-	-	4.68	46.22	-	-
BIRD CHL137	-	-	-	55.63	-	-
BIRD BAC402	0.22	1.53	8.59	1.53	-	-
BIRD BAC430	-	-	0.83	3.50	14.46	1.79
BIRD BAC431	0.27	**3.58**	-	-	**14.79**	**1.21**
BIRD BAC464	0.23	0.44	0.53	3.22	11.11	1.05

Screening the effect of different components of modified Navicula nutrient medium on biomass production of the isolate BIRD BAC431 (*Phaeodactylum tricornutum*) by Plackett-Burman

The significant effect of different nutrients on the growth and EPA production by the isolate *Phaeodactylum tricornutum* were explored using the generated PB matrix. The responses to be measured includes dry weight, EPA%, EPA yield (mg/g) and EPA concentration (mg/L) as shown in **Table 3**. The experimental runs were performed in a randomized order and the maximum and minimum levels used for each variable are indicated as (+) and (-), respectively. The highest growth of isolate BIRD BAC431 (*Phaeodactylum tricornutum*) was achieved in trial No.2 (1.055 g/L) while the highest amount and productivity of EPA were obtained in trial No.13 (4.33 mg/g and 1.944 mg/L respectively).

The experimental responses were subjected to the analysis of variance and the parameter estimates and results are summarized in **Table 4**. The *P* value designates a statistical confidence of a factor estimate. A *P* value of <0.05 was used as a cut-off point indicating the statistical significance of a factor at 95 % confidence level.

Pareto charts in **Fig. 1** also show the effect of each of the tested variables where the plotted values are arranged from the most significant factor to the smallest.

The factors located on the right of the reference line are significant factors and those located on the left of it are non-significant factors.

Among all factors sea salt showed the highest significant effect on all responses whereas $Na_2EDTA.2H_2O$ showed a non-significant effect on all responses.

According to the results, the recommended modified medium composition to achieve the maximum productivity was (0.05 g/L $Ca(NO_3)_2.4H_2O$, 0.21 g/L $K_2HPO_4.3H_2O$, 0.0125 g/L $MgSO_4.7H_2O$, 0.05 g/L $Na_2SiO_3.9H_2O$, 0.01g/L Na_2CO_3, 0.0075g/L $FeCl_3.6H_2O$, 0.045g/L $Na_2EDTA.2H_2O$, 4.2 mg/L H_3BO_3, 0.45 mg/L $MnCl_2.4H_2O$, 0.0625 mg/L $CuSO_4.5H_2O$, 0.04 mg/L $HMoO_4$, 0.135 mg/L $ZnCl_2$, 0.0615 mg/L $CoCl_2.6H_2O$, 10 g/L sea salt, 1.275 mg/L $Na_2SeO_3.5H_2O$, 50 µg/L $AlCl_3$, 0.2M glycerol and 9 ml of *Spirulina* filtrate).

A subsequent validation experiment was carried out using the recommended medium composition and the amount of growth achieved was (1.048 g/L) and the amount of EPA was (14.5 mg/g) after 10 days of incubation (**Table 5**).

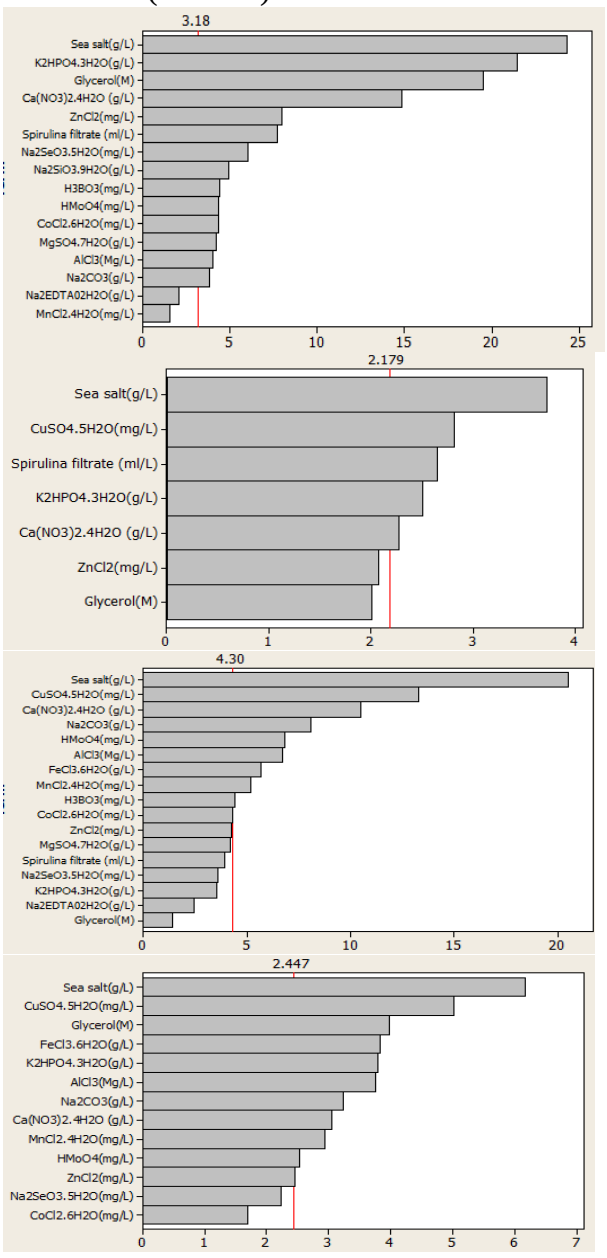

Fig. 1: Pareto charts of the standardized effect to show the significance of each factor on each

eicosapentaenoic acid (EPA) response of *Phaeodactylumtricornutum*(A) dry weight(g/L) (B)EPA %, (C) EPA yield (mg/g), and (D)EPA concentration (mg/l).

Table 5: Productivity of Phaeodactylum tricornutum grown for 6, 8 and 10 days in its optimized medium compared to its control medium.

Response	Control medium	Result(6^{th}day)	Result(8^{th} day)	Result(10^{th} day)
Dry weight	1.054	0.738 g	0.82 g	1.048 g
EPA yield	1.6	6.59 mg/g	12.467mg/g	14.5mg/g
EPA concentration	1.69	4.86 mg/L	10.22mg/L	15mg/L
EPA %	18.88	12 %	10.5%	10.757%

Table 3: The matrix and the responses for the screening Plackett–Burman design for *Phaeodactylum tricornutum*

Run	Variables																		Responses				
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	X_{18}	X_{19}	Dry Wt.(g/L)	EPA %	EPA yield (mg/g)	EPA conc. (mg/L)
1	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	0.606	0.653	0.43	0.26
2	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	1.055	1.366	0.49	0.517
3	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	0.697	4.554	2.6	1.812
4	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	0.799	4.633	1.93	1.542
5	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	0.521	4.085	2.040	1.063
6	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	0.815	2.529	0.68	0.554
7	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	0.823	2.99	1.37	1.128
8	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	0.589	3.151	1.18	0.695
9	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	0.57	4.902	1.77	1.009
10	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	0.439	1.699	0.599	0.263
11	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	0.504	1.28	0.42	0.212
12	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	0.629	1.759	0.64	0.403
13	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	0.449	4.51	4.33	1.944
14	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	0.583	1.035	0.5	0.292
15	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	0.634	1.729	0.38	0.241
16	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	0.401	3.132	1.14	0.457
17	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	0.566	2.57	2.15	1.217
18	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	0.994	2.474	0.56	0.556
19	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	-	0.833	3.413	1.05	0.874
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.258	1.593	2.46	0.634
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.539	2.55	2.56	1.379

Table 4: Estimated effects, coefficients and *p* values for the tested variables for *Phaeodactylum tricornutum*

Variables	EPA yield (mg/g)				EPA concentration (mg/L)				EPA %			
	Effect	Coefficient	T value	*P* value	Effect	Coefficient	T value	*P* value	Effect	Coefficient	T value	*P* value
$Ca(NO_3)_2.4H_2O$	-0.874	-0.437	-4.69	**0.018**	-0.361	-0.1805	-7.31	**0.018**	-0.7508	-0.3754	-2.27	**0.043**
$K_2HPO_4.3H_2O$	0.8324	0.4162	4.47	**0.021**	0.6196	0.3098	12.55	**0.006**	0.8284	0.4142	2.50	**0.028**
$MgSO_4.7H_2O$	0.1292	0.0646	0.56	0.673	0.3804	0.1902	7.70	**0.016**	0.3518	0.1759	0.56	0.676
$Na_2SiO_3.9H_2O$	0.7748	0.3874	4.16	**0.025**	0.3188	0.1594	6.46	**0.023**	0.1956	0.0978	0.31	0.809
Na_2CO_3	-.3004	-0.1502	-1.61	0.205	-.1234	-0.0617	-2.50	0.130	-0.1372	-0.0686	-0.22	0.864
$FeCl_3.6H_2O$	0.87	0.435	4.67	**0.019**	0.6184	0.3092	12.52	**0.006**	0.2452	0.1226	0.39	0.764
$Na_2EDTA.2H_2O$	0.6952	0.3476	3.73	**0.034**	0.3546	0.1773	7.18	**0.019**	0.4044	0.2022	0.64	0.637
H_3BO_3	0.524	0.262	2.81	0.067	0.0834	0.0417	1.69	0.233	0.5312	0.2656	0.84	0.555
$MnCl_2.4H_2O$	0.1872	-0.0936	-0.83	0.563	-0.238	-0.119	-4.82	**0.040**	-0.2754	-0.1377	-0.44	0.738
$CuSO_4.5H_2O$	-.5732	-0.7866	-8.45	**0.003**	-.8268	-0.4134	-16.74	**0.004**	-0.9324	-0.4662	-2.82	**0.016**
$HMoO_4$	-.7906	-0.3953	-4.24	**0.024**	-.4338	-0.2169	-8.78	**0.013**	-0.1652	-0.0826	-0.26	0.837
$ZnCl_2$	0.236	0.118	1.27	0.295	0.0958	0.0479	1.94	0.192	0.6862	0.3431	2.07	**0.060**
$CoCl_2.6H_2O$	0.4768	0.2384	2.56	0.083	0.0914	0.0457	1.85	0.205	-0.1514	-0.0757	-0.24	0.850
Sea salt	1.7772	-0.8886	-9.54	**0.002**	-0.757	-0.3785	-15.33	**0.004**	-1.2318	-0.6159	-3.72	**0.003**
$Na_2SeO_3.5H_2O$	0.6438	0.3219	3.46	**0.041**	0.3826	0.1913	7.75	**0.016**	0.2322	0.1161	0.37	0.776
$AlCl_3$	0.8332	0.4166	4.47	**0.021**	0.5644	0.2822	11.43	**0.008**	0.2206	0.1103	0.35	0.786
Glycerol	0.3076	0.1538	1.65	0.197	0.528	0.264	10.69	**0.009**	0.6622	0.3311	2.00	0.069
Spirulina filtrate	-0.262	-0.131	-1.41	0.245	0.0464	0.0232	0.89	0.537	0.8744	0.4372	2.64	**0.021**

Discussion

Long-chain polyunsaturated fatty acids (LC-PUFAs), especially eicosapentaenoic acid (EPA) is important for human health as they play indispensable role in preventing cardiovascular and cancer diseases and act as precursors of a group of eicosanoids, hormone-like substances such as prostaglandins, thromboxanes and leucotrienes that are crucial in regulating developmental and regulatory physiology [18].

LC-PUFAs are synthesized by desaturases and elongation enzymes which convert short saturated fatty acids to long unsaturated fatty acid. Mammals and human bodies lack the ability to synthesize linoleic acid (LA, n-6), and α-linoleic acid (ALA, n-3) as they have not the desaturase necessary to synthesize them. ALA and LA are the precursors of other LC-PUFAs [19]. Therefore food should contain these essential FAs, which is given by eating fish or vegetables daily. Fish cannot synthesize the LCPUFAs de novo, but taking them from marine microalgae they consume [20].

Microalgae are photoautotrophic organisms that can synthesize a variety of important compounds such as carotenoids, vitamins and lipids using CO_2 and solar energy. They are known as the primary producers of LC-PUFAs in aquatic environments [21, 22, 23, 24, 25]. A pivotal requirement for microalgae as a source of polyunsaturated fatty acids is not only high content of desirable fatty acids, but also biomass production on a sustainable and cost-competitive basis. With fast growth rates and low production costs relative to other organisms, microalgae provide useful cell factories for LC-PUFAs production [26, 27, 28, 29, 30].

Nine marine microalgae isolates were obtained from the culture collection of Biotechnology International Research and Development Centre (BIRD), Mansoura, Egypt as test marine microalgae for production of long chain polyunsaturated fatty acids (LC-PUFAs).These isolates were screened for their potential PUFAs production. The isolates were screened by means of gas chromatography/mass spectroscopy for accurate identification of the extracted FAME. Eight isolates namely (*Chlorella sorokiniana* (BIRD CHL120), *Chlorella salina*(BIRD CHL125), *Dunaliella salina* (BIRD CHL136), *Dunaliella salina*(BIRD CHL137), *Amphora marina*(BIRD BAC402), *Phaeodactylum tricornutum* (BIRD BAC430, BIRD BAC431, BIRD BAC464)) were able to synthesize and produce different types of PUFAs. The major producers of EPA were belonged to Bacillariophyta. In addition LA and ALA were found to be abundant in the members of Chlorophyta.

The highest producer of EPA, among the test isolates, the pinnate diatom *Phaeodactylum tricornutum* [31, 32, 33]. This result is; as they found that diatoms and dinoflagellates are the most potential source of EPA and DHA within algal world. Also Yongmanitchai and Ward [34], Martins *et al*. 2013 [35] reported that *Phaeodactylum tricornutum* to be a good PUFAs producer especially EPA.

Accordingly; the isolate *Phaeodactylum tricornutum* was chosen as target species to be optimized; due to its advantages in growth rate and long chain polyunsaturated fatty acids (LC-PUFAs) content (14.8 % EPA, 1.2% DHA).

The analysis of the Plackett-Burman screening experiment showed that two variables have the most significant effect on all the EPA responses namely NaCl and $Ca(NO_3)_2.4H_2O$. Glycerol showed significant effect on the growth and EPA concentration.

NaCl was found to have a statistically significant effect on *Phaeodactylum tricornutum* productivity of EPA with the minimum concentration (10 g/l) leading to higher production. The high concentration of NaCl has negative effect on DHA production of *Crythecodinium cohnii* ATCC 30556, with a significant decrease in DHA produced when increasing the concentration of NaCl from 15 to 30 g/l in the cultivation media [36]. The properties of membranes are related to the fluidity of the constituent lipids. In particular, salinity changes can induce elongation and desaturation of FAs chains to allow osmoregulation in microalgae [37]. $Ca(NO_3)_2.4H_2O$ also has a significant effect on EPA production in the tested microalga with the minimum concentration (0.05 g/l) leading to higher production. Deng *et al*.; 2011 [38] showed that lipid production by *Chlorella*

vulgaris significantly increased when grown in Calcium and Magnesium free medium. In addition, higher oil content obtained under nutrient starvation conditions, especially nitrogen deficiency [39, 40].

Glycerol showed significant effect on the growth and EPA concentration with the maximum concentration (0.2 M). *Phaeodactylum tricornutum* has the ability to grow mixotrophically and can grow on glycerol, acetate, glucose and fructose [41, 42, 43]

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