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Growth promoting bacteria as value added in productivity of microalgae

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Abstract: *Chlorella vulgaris* was cultured on Bold's Basal Medium (BBM). Three bacterial isolates coded as P3, P4 and P7 were isolated on Nutrient Broth medium (NB). Based on analysis of 16S rDNA of bacterial isolates, they were found to be contiguous similarity to *Bacillus amyloliquefaciens*, *Pseudomonas rhizosphaerae,* and *Pseudomonas aeruginosa*, respectively. C. *vulgaris* was cocultured with each bacterial strain at different concentrations, and it was found that the P4 bacterium had a growth-promoting effect on *C. vulgaris, causing* cell count to be increased six times greater than that of *C. vulgaris* axenic culture. Indole acetic acid (IAA) productivity was estimated for three strains of bacteria. It was found that the P4 strain has superiority in IAA productivity during culturing in NB containing Tryptophan compared to NB and recycled BBM (1.156±0.0097µg/ml, 1.064±0.0045µg/ml and 1.033±0.003 µg/ml), respectively. In contrast, P4 never produced IAA in fresh BBM medium. Furthermore, it was found that IAA produced by *C. vulgaris* upgraded to 0.987±0.0117 µg/ml. Moreover, it was found the highest cell number of *C. vulgaris* growing in coculture with P4 (1 algae:5 bacteria as v:v), although the highest culture density was investigated in the supernatant. Also, biomass productivity was highest in the supernatant than in P4 (1algae:5bacteria) 0.059±0.002 and 0.035±0.003 g/l/day, respectively. The highest ratio of chlorophyll a and chlorophyll b was in algae treated with P4 strain (1 algae:5 bacteria as v:v) recorded 2.447and 1.887 µg/ml respectively. Interestingly, the lipid productivity by *C. vulgaris* was the highest in the presence of IAA compared to axenic culture, being 1.608±0.124 f 1.176±0.164 g/l/day, respectively.

keywords: Microalgae, Coculture,*Chlorella* , *Pseudomonas sp.*

1.Introduction

Algae are the most important primary producers in the aquatic ecosystem, accounting for more than half of all global net primary productivity**(1)**. Microalgae are utilized for various applications, including the creation of particular colors and the biological treatment of wastewater for ammonia, phosphorus, and heavy metals**(2)**. They have a range of size from microscopic unicellular algae such as cyanobacteria and diatoms to gigantic multicellular macroalgae such as giant kelp. Algae are primary producers, by which they transform carbon dioxide into organic compounds, which are then decomposed and recycled by the heterotrophic consumer organisms**(3)**. They are an excellent biofuel feedstock because of their high growth rates, ability to grow in inhospitable land and water (deserts, wastewaters, salt waters), and ability to produce a diverse range of fuels and byproducts (diesel, jet fuel, hydrocarbons, biogas, ethanol, feed, fertilizer, nutraceuticals and pharmaceuticals)**(4)**. Microalgae and bacteria are among the world's most primitive species, capable of adapting to various circumstances. While microalgae are photosynthetic, most bacteria are heterotrophic and can exist in oligotrophic settings. This is because algae and bacteria cooperate to adapt to changing conditions, even though the relevance of algae-bacteria interactions is unclear**(5)**. The interaction between algae and bacteria in 3 types 1: Mutualism is a biological interaction in which two or more species benefit from one another, 2: commensalism is a partnership in which only one party benefits and 3: Parasitism

is one species benefits at the expense of the other and exerts negative effects on it**(6)**.

However, scaling up the manufacture of biofuels from algal lipids is hampered by productivity concerns. Lipid accumulation in nutrient-depleted circumstances is a critical factor in algal monoculture success. In contrast, the stress circumstances reduce the biomass productivity of the treated alga**(7)**. The non-polar lipids [acylglycerols, sterols, free (non-esterified) fatty acids, hydrocarbons, wax, and steryl-esters] and polar lipids are the two main categories of lipids found in microalgae (phosphoglycerides, Glycosylglycerides). Non-polar lipids, particularly triacylglycerols (TAG), are plentiful storage products that can be rapidly catabolized to generate metabolic energy as a result many algae accumulate in it in large concentrations, reaching up to 20%–50% of cell dry weight**(8).**

This study aims to increase dry biomass and/or lipid productivity by coculture with bacteria. Bacteria-algae consortia and cocultures are two methods for increasing algal biomass with symbiotic or mutualistic bacteria. Significant biomass stimulation has been observed in artificially produced algal consortia with growth-promoting bacteria. This paper studied algal co-cultivation with bacterial pellets to enhance biomass or algal cultures' lipid productivities.

1.Materials and methods

2.1. Microorganism, isolation, purification and identification

2.1.1.Purification of test microalga

The alga was obtained from the algal collection in the algal biotechnology and water quality lab, Faculty of Science, Mansoura University, Egypt.

Under the aseptic condition, the microalgae was subcultured on sterilized solid Bold's Basal Medium (BBM) and incubated at 26˚C, 35.1 µ.mol/.m^2/s^1 cool white fluorescent lamps for seven days and re-streaked several times until pure axenic culture was obtained. A single colony was inoculated in 5 ml autoclaved liquid BBM and incubated at 26˚C, 35.1 µ.mol/m^2/s^1cool white fluorescent lamps with light/dark cycle 16h/8h and continuous sterilized (0.22 µm filter) air left mixing. The microalga was examined under a light microscope, and its shape characteristics features were used to identify the alga according to the morphological identification keys **(9).**

2.1.1.Algal growth curve

Growth curve estimation has proceeded as follows; 180 ml BBM medium in each Erlenmeyer flask (250ml vol) in triplicate. Each flask was inoculated with 20 ml algal inoculum (OD= 3.69). All flasks were incubated at 26˚C, 35.1 µ.mol/m^2/s^1 cool white fluorescent lamps with light/dark cycle 16h/8h and continuous sterilized (0.22 µm filter) air left mixing. During the experiment period (eight days), the culture density was measured using a spectrophotometer at 440nm, and cells were counted every 24 h.

2.1.Bacteria

2.1.1.Cultivation, preservation and purification of bacteria

Three isolates of bacteria kindly provided by the Plant Physiology lab were cultivated in 50 ml autoclaved nutrient broth medium (NB) and incubated with shaking (120 rpm) at 26°C and 35.1 µ.mol/m^2/s^1 for 48 h.

These strains were streaked on nutrient agar medium to confirm the purity

2.1.1.Molecular identification

Bacterial isolates were identified by analyzing the sequence of the 16S rRNA gene. Briefly, bacterial genomic DNA was extracted using a SolGent (Solutions For Genetic Technologies)purification kit, and 16S rRNA was amplified in PCR reaction using Solgent EF-Taq, PCR Machine 9700(ABI) as follows: 95°C 15min, 95°C 20 sec, 50°C 40 sec for 30 Cycles, 72°C for 90 sec then 72°C for 5 min. Universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') were used. PCR product was purified with Solgent PCR purification kit and sequenced by ABI 3730XL DNA Analyzer, Applied Biosystems, USA. The generated sequences were analyzed by Finch TV (version 1.4.0) software(10), and the phylogenetic tree was generated via Mega X software (11) using the closest published type strains sequences.2.1.1.Bacterial growth curve

Single colonies (48h) were inoculated in 50 ml NB medium at 26°C, 35.1 µ.mol/m^2/s^1 cool white fluorescent lamps with shaking at 120 rpm and culture O.D at 600 nm was measured for cell activation. After 24 h, 2ml from each isolate was inoculated in 200 ml NB medium and incubated at 26°C, 35.1 µ.mol/.m2/s1 cool white fluorescent lamps with shaking at 120 rpm, and the O.D was measured at 600 nm every one hour.

2.1.Determination of the optimum ratio of alga: bacteria coculture

Single colonies from bacterial isolates P3, P4, and P7 (colony age 48h) were inoculated in 50 ml autoclaved NB medium and incubated at 26°C, 35.1 µ.mol/m^2/s^1 cool white fluorescent lamps with shaking at 120 rpm for 24 h. Culture density was measured for each strain at 600 nm. The pellet of each isolate was prepared by centrifugation at 4000 rpm for 10 min **(12)** and washed by autoclaved BBM three times. These pellets were used for further experiments. . The tested ratio of algae: bacteria were 1:1, 1:5, and 1:10 based on culture density.

Bacterial pellet was resuspended by BBM medium and was inoculated into a 250 ml Erlenmeyer flask which contained 180 ml of BBM medium. Then 20 ml algal inoculum (OD= 0.440) which represented algae in log phase was incubated at same previous conditions for seven days. Culture density and count were measured at zero time and every 24 h using a spectrophotometer at 440 nm. At the end of the experiment, at 4000 rpm the dry algal biomass was centrifuged for 10 min, washed three times with distilled water, centrifuged at 4000 rpm for 5 min, then dried in an oven at 60°C.

2.1.Estimation of indole acetic acid (IAA)

2.1.1.Estimation of IAA for bacteria in nutrient broth medium (NB) under cool white fluorescent lamps

Nutrient Broth (NB) medium (50 ml) and NB medium (50 ml) supplemented by 1.250 µg tryptophan were inoculated with the test bacteria. The cultures were incubated at 26°C, 35.1 µ.mol/m^2/s^1 cool white fluorescent lamps with shaking at 120 rpm for 24 hours.

After incubation, cultures were centrifugation at 4000 rpm for 20 min. Pellets and supernatants were separated. Pellets were resuspended in (NB) medium, then disrupted by ultrasonic for 15 min and centrifugated at 4000 rpm for 20 min. 2 ml of the supernatants were transferred to a test tube. Two drops of orthophosphoric acid were added, followed by 4 ml of Salkowski reagent (2.03 g ferric chloride ($FeCl_3$) dissolved in 500 ml distilled water and 300 ml concentrated sulphuric acid (H_2SO_4)), and were incubated for 25 min at room temperature. The development of pink color indicates indole acetic acid (IAA) production. The optical density was read at 540 nm**(13).** IAA content in each sample was determined according to a standard curve of authentic IAA (0 - 50 µg/ml).

2.1.2. Estimation of IAA for bacteria in fresh and recycled Bold's Basal medium (BBM) and for alga.

Fresh Bold's Basal Medium (BBM) and inoculated (BBM) medium were prepared and inoculated (BBM) medium (O.D = 1.635) was centrifugated at 4000 rpm for 10 min, and then the supernatant was transferred to autoclaved flask. Fresh (BBM) and supernatant of inoculated (BBM) medium were inoculated with P3, P4 and P7 with culture density (2.148,2.488 and 2.072, respectively). Cultures were incubated at 26°C, 35.1 µ.mol/m^2/s^1cool white fluorescent lamps with shaking 120 rpm for 24 h. After centrifugation, two ml of supernatant from each culture were transferred to a test tube and the IAA content was determined according to the method of (*Glickmann et al*., 1995).Also, the IAA content was determined in algal culture prepared in stationary phase (culture density = 1.635).

2.1.Algae coculture with P4 bacterium

Erlenmeyer flask (500ml) contain 360 ml of BBM medium was inoculated with 40ml algal inoculum (OD = 1.332) as a control. Erlenmeyer flask (500ml) containing 360 ml of BBM medium was inoculated with 40ml algal inoculums and a pellet of bacteria volume 40 ml as (1 algae: 1 bacteria). Erlenmeyer flask (500 ml vol) containing 360 ml of BBM medium was inoculated by 40 algal inoculums and pellet of bacteria volume 200 ml as (1: 5). Erlenmeyer flask (500ml vol) containing 360

ml of BBM medium was inoculated by 40 algal inoculums and pellet of bacteria volume 400 ml as (1: 10). Further controls were prepared in which an Erlenmeyer flask (500ml vol) containing 360 ml of BBM medium was inoculated by 40 ml algal inoculum and 0.462 mg as (IAA). The supernatant of bacteria P4 (400 ml) inoculated in Erlenmeyer flask (500ml vol) with stocks of BBM medium was inoculated by a pellet of (360 ml BBM medium + 40 ml algal culture (O,D = 1.332)) which final O,D (0.148) as (Supernatant).

All cultures were prepared in triplicate and incubated at 26°C, 35.1 µ.mol/m^2/s^1 cool white fluorescent lamps with light/dark cycle 16h/8h with continuous sterilized (0.22 µm filter) air left mixing for seven days.

Cell count and culture density (440 nm) was measured every day. At the end of the experiment, algal cultures were harvested by centrifugation for 10 min at 4000 rpm, then washed very well and centrifugated again at 4000 rpm for 5 min, and pellets were dried at 60 °C in an oven.

2.1.1.Chlorophyll assay

Fresh culture (5 ml) was centrifugated at 4000 rpm for 10 min, and pellets were taken and resuspended in acetone and left overnight in the dark for pigment extraction. The OD of all extracts was measured at 470, 653 and 666nm. Pigments content was calculated using the following equations**(14)**

Chlorophyll a (Ch. a) = (15.65* $O.D_{666}$) – (7.340* $O.D_{653}$)

Chlorophyll b (Ch. b) = (27.05* $O.D_{653}$) – (11.21 * $O.D_{666}$)

Xanthophylls and carotenoids =((1000* $O.D_{470}$) – (2.860*Ch. a) – (1292*Ch. b))/245

2.1.1.Lipid assay

Lipids were estimated by sulfo-phosho vanillin method: 0.02 g of dry algal biomass were extracted in a known volume of solvent (2 chloroform: 1 methanol) overnight. Fresh phospho- vanillin reagent was prepared (120 mg vanillin dissolved in 20 ml distilled water, and the total volume was raised to 100 ml by phosphoric acid). 100 µl from each extract was transferred into another clean glass tube and dried at 60°C in an oven. Then one ml of sulfuric acid was added to each tube, and the tubes were placed in a boiling water bath for 10 min. Then allowed to cool, and 2.5 ml phospho-vanillin reagent was added and incubated at 37°C for 15 min until pink color appeared.

Then O,Dwas measured spectrophotometrically at 530 nm. The lipid content was estimated using a standard curve of cholesterol (0-450 µg/ml) **(15)**

The lipid productivity was measured gravimetrically (gl^{-1} day^{-1}), along with the product of biomass productivity (gl^{-1} day^{-1}) and lipid content (% dry biomass).**(16)**

2. Results

2.1. Algae

2.1.1. Alga identification and growth characteristics

The microscopic examination of the test microalga showed a single nonmotile spherical cell, sometimes without a pyrenoid, with a diameter of 10 µm and had a parietal cup chloroplast which has been identified as *Chlorella vulgaris***(9)**. Figure(1) shows the algal cells under a light microscope.

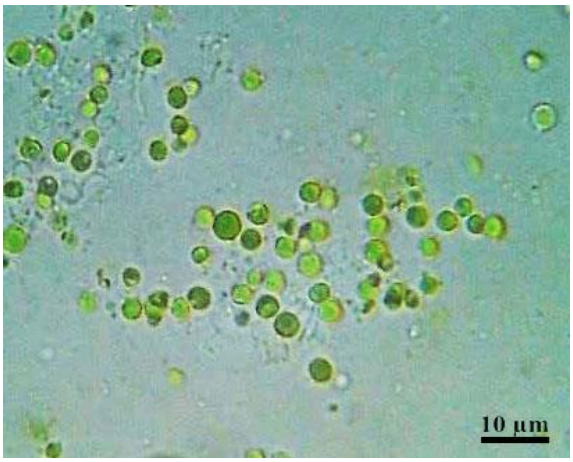

Fig (1): The *Chlorella* alga under the light microscope (400X) shows single-celled, nonmotile, spherical, and a diameter of 10 µm.

2.1.1.Growth characteristic of *Chlorella*

The growth characteristics of *Chlorella* was assessed by monitoring culture optical density at 440 nm and cell count as shown in Figure (2). It was found that culture density increased from the first to the fifth day. Cultural density increased significantly from day six until it reached the highest growth on day nine. However, the number of algae increased from the first day and reached the highest number on day seven which is shown in Figure (2).

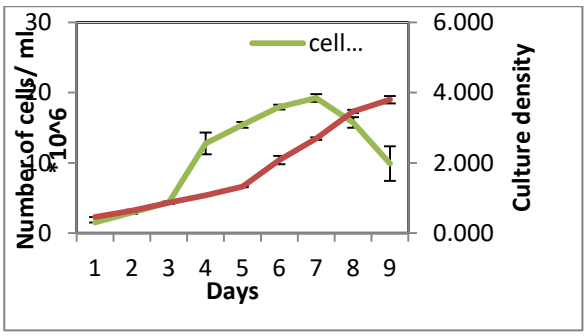

Fig (2): Culture density of *Chlorella* at 440 nm) and the number of *Chlorella* cells/ml grown on BBM at 26˚C 35.1 µ.mol/m^2/s^1 cool white fluorescent lamps with light/dark cycle 16h/8h with continuous sterilized (0.22 µm filter) air left mixing for nine days. The results are recorded as the Mean of triplicates ± Standard Error (S.E).

2.1.Bacteria

2.1.1.Bacterial purification and identification

The three bacterial colonies had spherical shapes, although the color of P3 and P7 colonies were white and P4 colonies color was yellow. The margins of P4&P7 were entire, unlike that of P3. Only bacterial strain P7 was pigmented (green color), as shown in Table(2) and Figure (3).

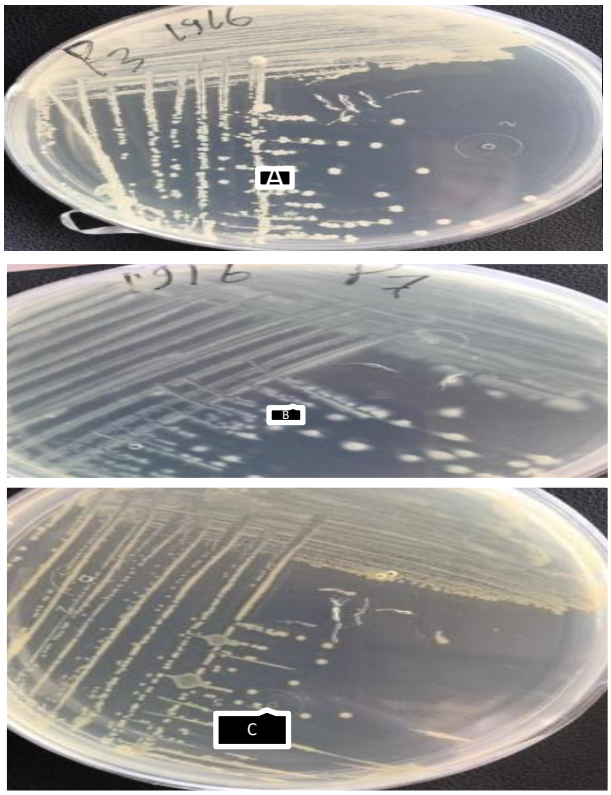

Fig (3): A, B & C referred to bacterial isolates P3, P7 & P4, respectively, grown on nutrient agar medium, pH seven after 48 h incubation in the dark at 26 ˚C.

Table(1): Morphological characters of the isolates P3, P4 & P7 colonies grown on nutrient agar media, pH 7 after 48 h incubation in the dark at 26 ˚C.

Isolates	Shape	Colour	Margin	Pigmentation inmedia
P3	spherical	white	irregular	Not showing
P4	spherical	Yellow	Entire	Not showing
P7	spherical	white	Entire	Showing (green)

2.1.1.Molecular identification of bacteria

All three bacterial isolates, namely P3, P4 and P7, were identified by The Means of 16s rRNA sequencing as *Bacillus* P3, *Pseudomonas* P4 and *Pseudomonas* P7 with accession numbers of OK47725.1, JX134622.1 and MT598026.1 respectively. The phylogenetic tree of each isolate is represented in **Error! Reference source not found.**.

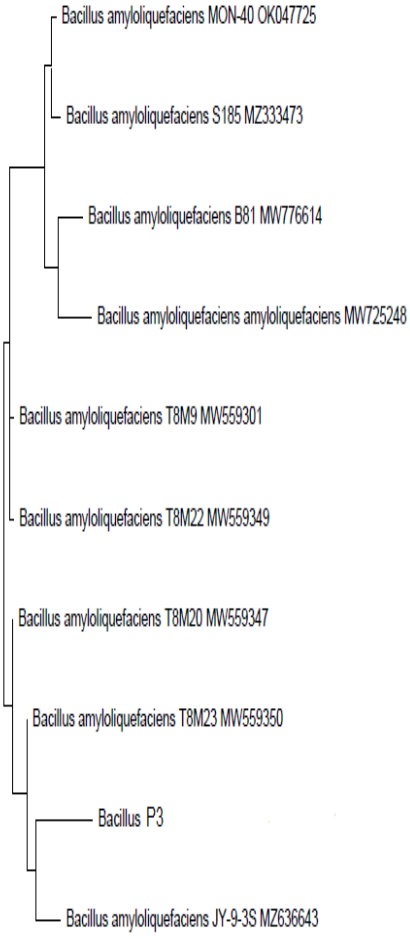

Fig (4): Phylogenetic tree of P3bacterial isolate showing closest similarity to *Bacillus amyloliquefaciens*

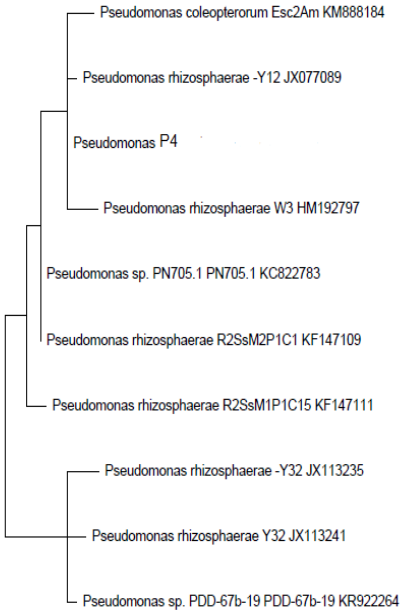

Fig (5): Phylogenetic tree of P4 bacterial isolate showing closest similarity to *Pseudomonas rhizosphaerae*

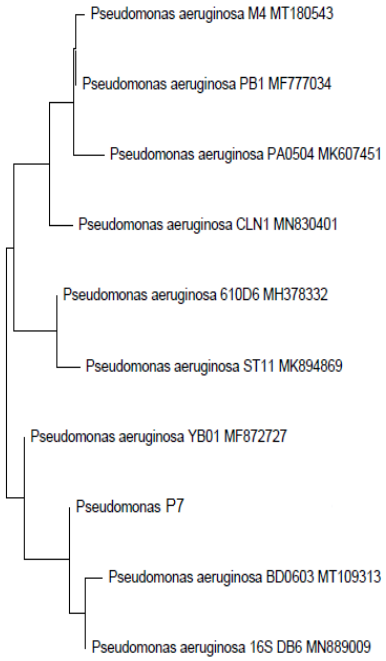

Fig (6): Phylogenetic tree of P7 bacterial isolate showing closest similarity to *Pseudomonas aeruginosa*

2.1.1.Bacterial growth curve under cool white fluorescent lamps

The tested strains were grown on a nutrient broth **(NB)** medium with shaking (120 rpm) at 26°C and 35.1 µ.mol/m^2/s^1 , and the culture density was measured after 24 h which means that the cells were able to grow under light conditions paving the way for their use with algae and show that culture density is still containing the growth as culture density measure. This is the initial step in determining the bacterial growth curve.

The three bacterial strains were inoculated in NB medium, and the growth was assessed by spectrophotometer at 600 nm. The culture density of the three bacterial strains increased almost steadily from zero time to 20 h. After 27 h, unstable growth was shown in Figure (7).

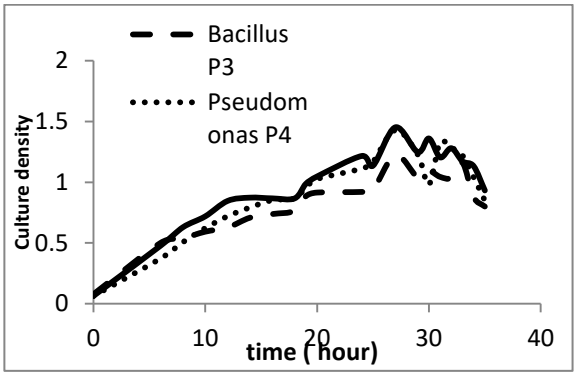

Fig (7): The culture density of the three bacterial strains P3, P4 & P7 at 600 nm incubated in NB medium, shaking (120 rpm) at 26°C and 35.1 µ.mol/m^2/s^1 for 35 h.

2.1.Determination of the optimum ratio of alga: bacteria coculture for algal growth

The lowest culture density was recorded in alga cocultured with P7 (1:10) (alga: bacteria), and cocultured alga with bacteria P4 strain (1:5) recorded the highest as recorded in Figure (8). The turbidity in coculture due to bacteria made the result of culture density not actual; therefore, it was necessary to determine the number of algae in each treatment.

The number of cells changed between treatments recorded as a small difference on the second day. On the third day, the number of cells in treatment P4(1:5) increased dramatically compared to the control, whereas the number of cells increased very little in the rest of the treatments. The number of *Chlorella* cells was still higher on the fourth day in treatment P4 (1:5) than in the other treatments and the control, which all were, to some extent, similar. On day five, the number of cells in treatments P4(1:5) was still the highest, but the number of *Chlorella* cells in treatments P3 (1:5) and P3 (1:10) increased by a large proportion compared to other treatments.

On day six, the number of *Chlorella* cells continued to increase in treatment P4(1:5),

unlike treatments P7(1:1) and P(1:10), which led to the least number of *Chlorella* cells. On the last day, the highest number of cells was recorded in treatment P4(1:5) as recorded in Figuer(9).

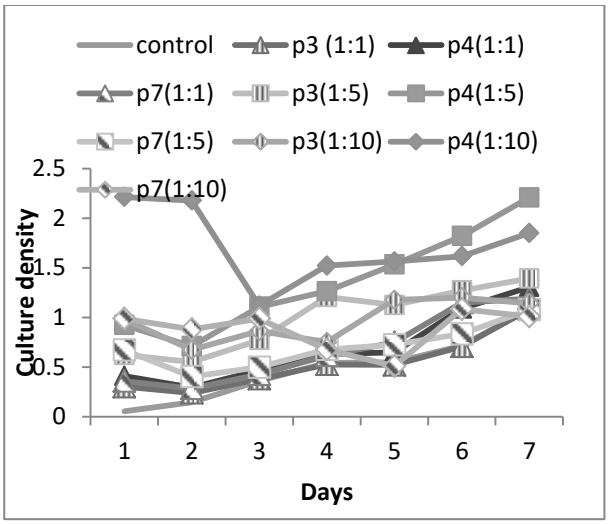

Fig (8): Growth characteristics of alga: bacteria coculture; with three ratios (1:1),(1:5) &(1:10). The P3*s*, P4 and P7 refer to bacteria strains.

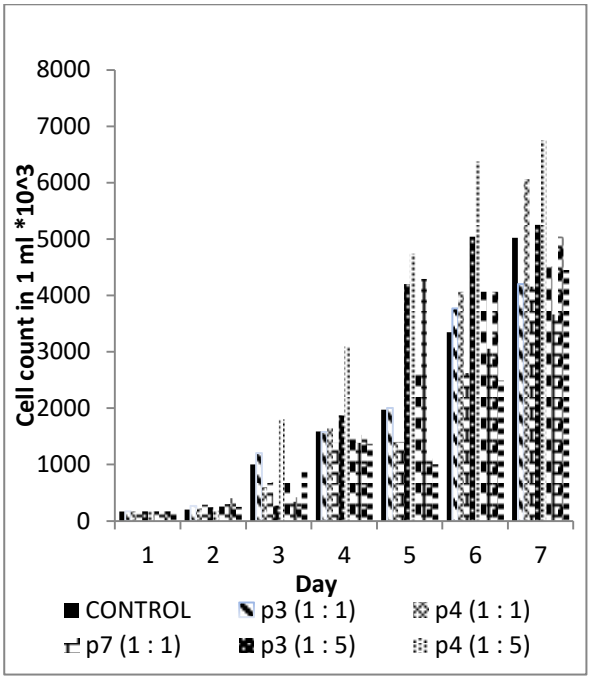

Fig (9):Cell number of *Chlorella* cocultured with different ratios of bacteria P3, P4= and P7 , (1:1) , (1:5) & (1:10) =(volume of alga: volume of bacteria

The algal biomass was collected by centrifugation at the end of the experiment, dried in an oven at 60°C, and weighed. Despite the lowest dry biomass observed for treatment P4 (1:10), the weight of dry biomass of treatment P4 (1:5) was the highest (0.35 g/l) when compared to the control and the obtained weights are shown in Figure (10).

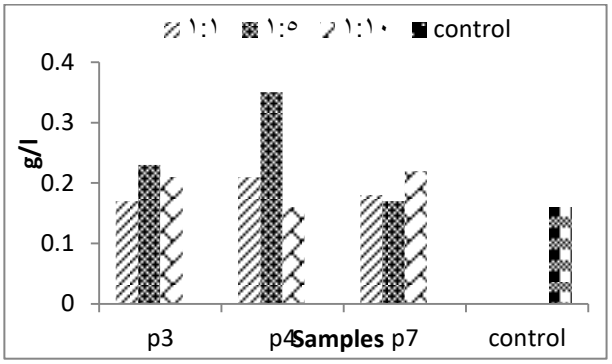

Fig (10):): Dry biomass of *Chlorella* cocultured with bacteria, P3, P4 and P7, in three ratios (volume of alga: volume of bacteria) (1:1), (1:5) & (1:10).

2.1.Estimation of indole acetic acid (IAA):

The three bacterial strain were inoculated in nutrient broth medium (NB), NB+ tryptophan, recycled Bold Basal Medium (BBM) and fresh BBM medium to study the amount of indole acetic acid (IAA) which were produced. Also, *Chlorella* was inoculated in fresh BBM medium to know its ability the production of IAA.(IAA)were measured as shown in Table (2). Upon investigating the bacteria's ability to produce indole acetic acid (IAA), P4 produced the highest IAA concentrations compared with P7 and P3.

Table (2): Concentration of IAA of three bacterial strains P3, P4 and P7 cultured in NB, NB+Tryptophan, recycled BBM medium and fresh BBM medium. Different superscript letters refer to significant variation with Duncan's test at P = 0.05 using SPSS software

Strains	NB(Control)	NB+Trptophn	Recycled BBM(Algae medium)	Fresh BBM
	IAA(µg/ml)	**IAA(**µg/ml)	**IAA(**µg/ml)	**IAA(**µg/ml)
P3	1.048^{a}± 0.0035	1.049^{a}± 0.0033	0.996^{a}±0.0018	No growth
P4	1.064^{b}± 0.0045	1.156^{b}± 0.0097	1.033^{b}±0.0031	No growth
P7	1.072^{b}± 0.0036	1.082^{c}± 0.0047	0.993^{a}±0.0018	No growth
Chlorella	NA	NA	NA	0.987± 0.0117

2.1.Biomass, pigment and lipid production of algae in coculture with P4 bacteria strain

In previous experiments, *Pseudomonas rhizosphaerae* strain was the best result in indole acetic acid(IAA) production and in

cocultured with alga. In this experiment, alga coculture with pellets of P4 bacteria strains in three ratios (volume of alga: volume of bacteria) (1:1), (1:5) and (1:10), alga cocultured with indole acetic acid (IAA) and alga cultured in the supernatant with BBM medium stocks. Both *Chlorella* and bacteria were inoculated in BBM medium at 26°C,35.1 µ.mol/m^2/s^1 cool white fluorescent lamps with light/dark cycle 16h/8h with continuous sterilized (0.22 µm filter) air left mixing for seven days and the maximum growth measured at 440 nm.

The evolution in culture density during the incubation period was somewhat similar for all treatments except algae cultured in supernatant of P4 bacteria strain, as shown in Figure (11).

The number of cells changed between treatments was a little difference between *Chlorella* and *Chlorella* in supernatant on the second and third days, although *Chlorella* in supernatant did not show a significant difference in cell count.

On the fourth day, *Chlorella* treated with IAA had a higher number of cells than the control and other treatments. Although this increase was not large, it showed significance. *Chlorella* treated with P4 strain (1:5) had a maximum increase on the fifth day, but it was not significant to *Chlorella* treated with bacterial strain P4 (1:10). Control was almost equal in number with *Chlorella* treated with bacterial strain P4 (1:1).

On day six, all treatments had no significant increase to each although, *Chlorella* treated with P4 strain (1:5) keep up the best result as a large number of alga cells, this significant showed in last day in cells number for treatment *Chlorella* treated with P4 strain (1:5) as shown in Figure (12).

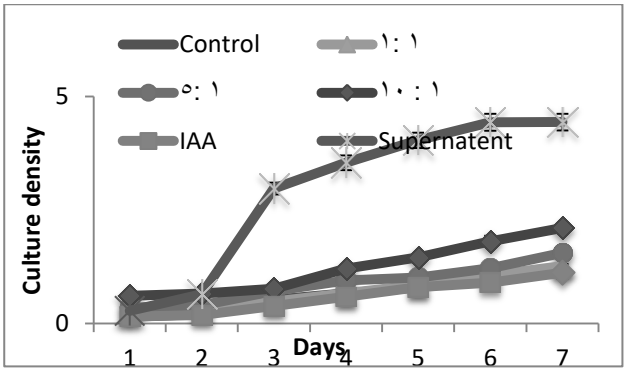

Fig (11):): Culture density measured at 440nm for *Chlorella* (control) and treatments, *Chlorella* cocultured with P4and with three ratios volume of alga: volume of bacteria (1:1),(1:5) &(1:10), *Chlorella* cocultured with indole acetic acid(IAA) and *Chlorella* with supernatant of P4 strain. The results are recorded as The Mean of triplicates ± Standard Error (S.E).

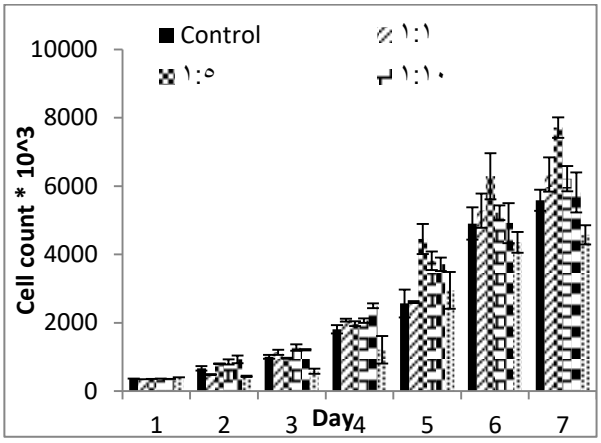

Fig (12): Number of algal cells/ml for *Chlorella* (control) and treatments, *Chlorella* cocultured with P4and with three ratios volume of alga: volume of bacteria (1:1),(1:5) &(1:10), *Chlorella* cocultured with indole acetic acid(IAA) and *Chlorella* with supernatant of P4 strain. The results are recorded as The Mean of triplicates ± Standard Error (S.E).

2.1.1. Dry algal biomass

At the end of the experiment, the algal mass was collected by centrifugation and dried in an oven at 60 ˚C, and the weight was recorded as showed in Figure (13).

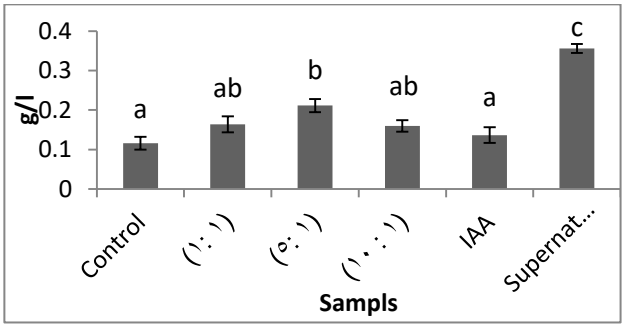

Fig (13): Dry alga biomass of *Chlorella* and *Chlorella* with treatments. The results are recorded as The Mean of triplicates ± Standard Error (S.E). Different superscript letters refer to significant variation with Duncan's test at P = 0.05 using SPSS software.

2.1.1.Chlorophyll assay

Amounts of chlorophyll a and chlorophyll b were recorded on the last day of the experiment for each treatment. The highest ratio of

chlorophyll a and chlorophyll b was in alga treated with P4 with a ratio (volume of alga: volume of bacteria) (1:5) recorded (2.446753 & 1.886737 µg/ml) respectively. There was no significant difference in the ratio of chlorophyll a, chlorophyll b and carotenoids in *Chlorella* treated with P4 (1:1), (1:10), and *Chlorella* treated with IAA. Treated alga with supernatant (supernatant) recorded the lowest chlorophyll aand chlorophyll b, as shown in Figures (14).

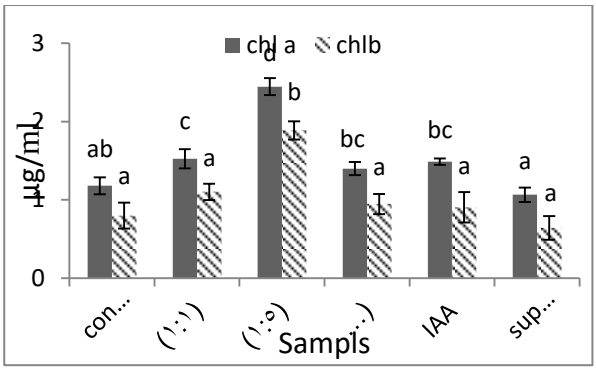

Fig (14): Chlorophyll a and chlorophyll b content in alga cocultured with P4 bacteria strain three ratios of alga: volume of bacteria (1:1),(1:5) &(1:10), treated *Chlorella* with indole acetic acid and treated *Chlorella* with supernatant of P4 strain the results are recorded as The Mean of triplicates ± Standard Error (S.E). Different superscript letters refer to significant variation with Duncan's test at P = 0.05 using SPSS software.

2.1.1. Lipids assay

Lipids content was estimated as well by sulfo-phospho-vanillin method in each treatment. The lipids content increased in treatment of alga cocultured with indole acetic acid (IAA),in which the highest lipids content was quantified gravimetrically as shown in Figure (15)

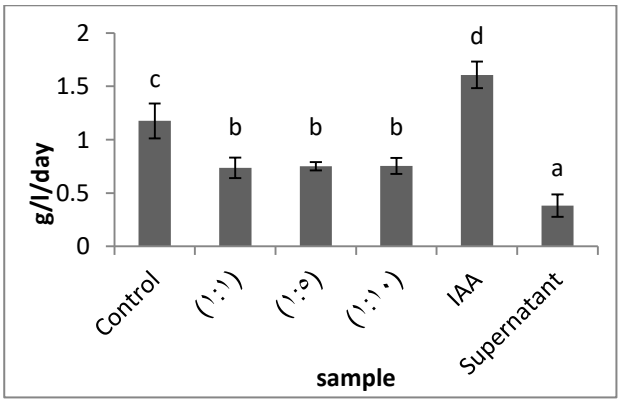

Fig (15): Lipid productivity (g/l/day) content in alga cocultured with P4 bacteria strain three ratios of alga: volume of bacteria (1:1),(1:5) &(1:10), treated *Chlorella* with indole acetic acid and treated *Chlorella* with supernatant of P4 strain the results are recorded as The Mean of triplicates ± Standard Error (S.E). Different superscript letters refer to significant variation with Duncan's test at P = 0.05 using SPSS software.

2. Discussion

The isolated *Chlorella* sp. was scaled up and grown in nutrient medium BBM because in many studies **(17)** was found the highest biomass concentration of *Chlorella Vulgaris, Chlorella minutissima* and *Chlorella sp* were in BG-11, followed by BBM but maximum lipid content and productivity was found in BBM media for all the *Chlorella* species. The isolated *Chlorella* sp was cultivated in BBM medium, the stationary phase on the seventh day and after that, growth decreased because of limiting nutrients or the rate at which the final yield is reached **(18&19).**

Bacterial growth usually was in the dark incubator **(20)**, but in this study work, isolated bacteria were incubated shaking(120 rpm) at 26˚c and 35.1 µ.mol/m^2/s^1 cool white fluoresce lamps to adapt to the conditions of algal growth. The stationary phase was in 24 h, and culture density became unstable, which under less favorable conditions, competition for nutrients increases and the cells become less metabolically active**(21).**

The present study focused on screening for different characteristics in the three isolated bacteria and the alga in terms of enhanced *Chlorella* growth from these symbiotic associations. Also, in this study, we provide further information on the relationship between *Chlorella* and *Pseudomonas rhizosphaerae* especially. Three isolates, P3, P4 and P7, belonged to *Bacillus amyloliquefaciens*, *Pseudomonas rhizosphaerae* and *Pseudomonas aeruginosa*, respectively.

In this experiment (Determination optimum ratio of alga: bacteria coculture), alga and bacteria were cultured separately until the stationary growth period as in**(22)**, then alga bacteria coculture with air left mixing without the impacts of shaking strengthened the bacterium-algae connection **(16)**, although in

some studies mixture of alga and bacteria incubated with shaking(**2)**.

The highest culture density, the number of alga and biomass were observed in bacterial strain P4 (1:5) ratio. In each ratio, the highest number of alga was bacterial strain P4, then P3 after that P7. The culture density is inaccurate because of turbidity, which bacterial pellet occurred in media. This difference is due to depending on the organic chemicals released. Because each phycosphere has a unique microenvironment, this selective relationship is possible**(5)**.

Mutualistic interactions between microalgae and heterotrophic bacteria have been observed, including the uptake by bacteria of extracellular organic carbon (EOC) produced by microalgal photosynthesis and the uptake by microalgae of the growth-promoting compounds produced by heterotrophic bacteria. Microalgae excrete organic matter during photosynthesis, including glucose, nitrogen, organic acids, lipids, and vitamins. Chlorella has also been found to excrete biotin, proline, and glycolic acid as extracellular organic matter ***(*23)**. Most green algae, especially *Chlorella*, are known to possess the ability to synthesize vitamins and phytohormones **(5)**. Also, the mechanism for increased algae development is suggested to be an exchange of algal DOC for bacteria-produced dissolved inorganic carbon (DIC) and sugars made available to algae. A major source of mineralized chemicals is the recycling of algal organic matter by bacteria. **(24)**.

Upon the molecular identification of the isolated bacteria, *Pseudomonas rhizosphaerae* was found to be reported as plant growth-promoting bacteria, and numerous studies have demonstrated the beneficial effects of coculturing plant growth-promoting bacteria with microalgae on a variety of factors, including the growth of algal biomass and the accumulation of specific metabolites**(5).**

The isolated bacteria P3(*Bacillus amyloliquefaciens)* gave somewhat good results as well as bacteria may induce negative effects on algal growth in several ways. Although, in other studies, interactions between *B. Anabaena* flosaquae 1092 and *Chlorella pyrenoidosa* 415 were found to be resistant to amyloliquefaciens T1 along with other cyanobacteria and green algae.**(25)**. Depending on the organic chemicals released, each phycosphere has a different microenvironment, resulting in such a selective relationship.

Bacteria can inhibit algal growth in a number of different ways **(5).** When bacteria receive signals from algae indicating cellular senescence, bacteria produce algaecides. Certain aquatic bacteria have the ability to lyse algal cells. For instance, protease, an algicidal protein released by the algicidal bacterium *Kordia algicida*, inhibits the growth of several marine diatoms. **(16**). The environment may have been rendered detrimental to algal growth by bacterial metabolism**(1)**. Bacteria might compete with algae for limiting nutrients, such as nitrogen and phosphorous when they grow together **(26)**.

The isolated bacteria P7 (*Pseudomonas aeruginosa*) coculturing with alga resulted in a very bad number of alga. *Pseudomonas aeruginosa* inhibits the growth of several green microalgae and cyanobacteria by producing agar-diffusible colored chemicals results that are consistent with those obtained in**(27)**. However, there was no information available regarding the inhibitor. Among other antibiotics, P. aeruginosa is known to produce glycolipids, pyo compounds, and phenazine pigments. But rather than having an antialgal effect, all of these compounds were discovered for their bactericidal activity. **(27)**. dry biomass was somewhat high because of this compound which bacteria produced in media.

Indole acetic acid (IAA) is one phytohormone that can begin the formation of the root and cause the growth and extension of plant roots. It has been demonstrated that exogenous IAA added to an algal culture encourages the growth of single-celled microalgae **(28&29).** Several microorganisms, particularly Plant Growth-Promoting Rhizobacteria, create IAA as a byproduct of L-tryptophan metabolism (PGPR) **(30).** While in plants, IAA is known to promote cell division and root elongation. **(16)**. In this study, IAA was produced in the presence of tryptophan also it was produced in the absence of tryptophan but in a small quantity, and these results are consistent with the result**(19)** in which Rhizobacterial strains were produced

auxin in the presence and absence of L-TRP although their potential varied a great deal, which L- Tryptophan (L-TRP) is considered an efficient physiological precursor of auxins in higher plants as well as for microbial biosynthesis of auxins. They observed a significant positive effect of L- TRP on growth parameters when applied at low concentration.

In this study, IAA was estimated for *Chlorella* and bacteria cultured in recycled BBM, and the results concerning IAA of *Chlorella* was lower than that obtained by bacteria. This is explained most green algae, especially Chlorella, are known to be able to synthesize vitamins and phytohormones **(5)**. IAA is the natural auxin commonly occurring in all vascular plants and in green algae from the *Chlorella* genus**(17)**. Bacteria can adapt and grow in different media, but this does not apply to all types of bacteria, such as P3 and P7, which have different characteristics.

In the second experiment (coculture with P4 bacteria strain), the number of *Chlorella* treated with exogenous IAA was higher than in control. This is in agreement with this study, where it was found IAA was the most effective in inducing culture growth in low concentrations (0.1µM) **(31)**.

In this study, *Chlorella* treated with supernatant of recycled NB medium produced the highest dry biomass, and this corresponds to the results in the search **(16)**.Which *Chlorella* treated with bacterial cell-free filtrate; NB - nutritional broth produced the highest dry biomass, although alga treated with a pellet of bacteria (1:1) not different from the control, contrary to this work which alga treated with a pellet of bacteria (1:1) gave small difference from control this difference almost doubled when alga treated with a pellet of bacteria (1:5).

Chlorella has grown positively in response to other *Pseudomonas spp* exudates, suggesting that bacterial glycoproteins may be significantly involved or that bacteria may provide algae with the vitamins they require to flourish. The reduction in algal O2 brought on by bacterial consumption and the resulting defense against oxygen damage are thought to promote algal growth. **(16)**.

Carotenoids and chlorophylls are components of the pigment composition in Chlorella species. The primary xanthophyll in both species was lutein. **(32)**. The highest value of chlorophyll a and b in alga treated with bacterial strain P4

Environmental stressors like salinity, temperature, heavy metal concentration, and, most importantly, nitrogen supply and increased light intensity have a significant impact on microalgal carotenoids production, which is species-specific and age-dependent. **(1)**.

In order to maximize lipid content, stress variables such as salts, temperature, light intensity, and growth phase have been taken into consideration, but not simultaneously with biomass. These factors all affect the accumulation of lipids. In most studies, treatments with high lipid content and low dry biomass were compared., according to **(16)**, and this is consistent with the results in this work. The average lipid content within the algae was 2% as reported by**(33)**. In terms of lipid productivity, the average observed lipid productivity is similarly comparable to values reported by others (2.6–38.7 mg L−1 day−1)**(34)**. *Chlorella* treated with exogenous indole acetic acid (IAA) gave the highest result than control in this work which IAA was considered a stressor, and this is a logical explanation for this result.

4. References

1 Ramanan, R., B.-H. Kim, D.-H. Cho, H.-M. Oh and H.-S. J. B. a. Kim (2016). Algae–bacteria interactions: evolution, ecology and emerging applications. **34**(1): 14-29.

2 Park, Y., K.-W. Je, K. Lee, S.-E. Jung and T.-J. J. H. Choi (2008). Growth promotion of Chlorella ellipsoidea by co-inoculation with Brevundimonas sp. isolated from the microalga. **598**(1): 219-228.

3 Kouzuma, A. and K. J. C. O. i. B. Watanabe (2015). Exploring the potential of algae/bacteria interactions. **33**: 125-129.

4 Hannon, M., J. Gimpel, M. Tran, B. Rasala and S. J. B. Mayfield (2010). Biofuels from algae: challenges and potential**. 1(5)**: 763-784.

5 Kim, B.-H., R. Ramanan, D.-H. Cho, H.-M. Oh, H.-S. J. B. Kim and Bioenergy (2014). Role of Rhizobium, a plant growth promoting bacterium, in enhancing algal biomass through mutualistic interaction. **69**: 95-105.

6 Fuentes, J. L., I. Garbayo, M. Cuaresma, Z. Montero, M. González-del-Valle and C. J. M. d. Vílchez (2016). Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds**. 14**(**5**): 100.

7 Chisti, Y. J. J. o. b. (2013). Constraints to commercialization of algal fuels. **167**(3): 201-214.

8 Borowitzka, M. A. and N. R. Moheimani (2013). Algae for biofuels and energy, Springer.

9 Wehr, J. D., R. G. Sheath and J. P. Kociolek (2015). Freshwater algae of North America: ecology and classification, Elsevier.

10 Idris, A. B., H. G. Hassan, M. A. S. Ali, S. M. Eltaher, L. B. Idris, H. N. Altayb, A. M. Abass, M. M. A. Ibrahim, E.-A. M. Ibrahim and M. A. J. I. J. o. M. Hassan (2020). Molecular phylogenetic analysis of 16S rRNA sequences identified two lineages of helicobacter pylori strains detected from different regions in Sudan suggestive of differential evolution. 2020.

11 Morimoto, Y., Y. J. Lu, H. Zuo, Z. Aibibula, M. Tohya, T. Kirikae, K. Hiramatsu, H. Daida and T. J. C. M. Baba (2021). Pseudomonas allokribbensis sp. nov. and Pseudomonas gozinkensis sp. nov., two new species isolated from a volcanic island, Izu Oshima, Japan. **78(4):** 1670-1677.

12 Converti, A., A. A. Casazza, E. Y. Ortiz, P. Perego, M. J. C. E. Del Borghi and P. P. Intensification (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. **48(6)**: 1146-1151.

13 Glickmann, E., Y. J. A. Dessaux and e. microbiology (1995). A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. **61**(**2**): 793-796.

14 Şükran, D., T. GÜNEŞ and R. J. T. J. o. B. Sivaci (1998). Spectrophotometric determination of chlorophyll-A, B and total carotenoid contents of some algae species using different solvents. **22**(**1**): 13-18.

15 Byreddy, A. R., A. Gupta, C. J. Barrow and M. J. J. o. m. m. Puri (2016). A quick colorimetric method for total lipid quantification in microalgae. **125**: 28-32.

16 Berthold, D. E., K. G. Shetty, K. Jayachandran, H. D. Laughinghouse IV, M. J. B. Gantar and bioenergy (2019). Enhancing algal biomass and lipid production through bacterial co-culture. **122**: 280-289.

17 Sharma, A. K., P. K. Sahoo, S. Singhal and A. Patel (2016). Impact of various media and organic carbon sources on biofuel production potential from Chlorella spp. 3 Biotech **6(2):** 116.

18 Richmond, A. (2008). Handbook of microalgal culture: biotechnology and applied phycology, John Wiley & Sons

19 Maier, R. M. and I. L. Pepper (2015). Bacterial growth. Environmental microbiology, Elsevier: 37-56.

20 Cho, K., J. Heo, D.-H. Cho, Q.-G. Tran, J.-H. Yun, S.-M. Lee, Y. J. Lee and H.-S. Kim (2019). Enhancing algal biomass and lipid production by phycospheric bacterial volatiles and possible growth enhancing factor. Algal Research **37**: 186-194.

21 Asghar, H. N., Z. A. Zahir, A. Khaliq and M. J. P. J. A. S. Arshad (2000). Assessment of auxin production from rhizobacteria isolated from different varieties of rapeseed. **37(3-4):** 101-104.

22 Yu, Q., P. Li, B. Li, C. Zhang, C. Zhang and Y. J. J. o. B. M. Ge (2022). Effects of algal–bacterial ratio on the growth and cadmium accumulation of Chlorella salina–Bacillus subtilis consortia. **62**(3-4): 518-529.

23 Watanabe, K., N. Takihana, H. Aoyagi, S. Hanada, Y. Watanabe, N. Ohmura, H. Saiki and H. J. F. m. e. Tanaka (2005). Symbiotic association in Chlorella culture. **51**(**2**): 187-196.

24 Le Chevanton, M., M. Garnier, G. Bougaran, N. Schreiber, E. Lukomska, J.-B. Bérard, E. Fouilland, O. Bernard and J.-P. J. A. R. Cadoret (2013). Screening and selection of growth-promoting bacteria for Dunaliella cultures. **2(3):** 212-222.

25 Yu, J., Y. Kong, S. Gao, L. Miao, P. Zou, B. Xu, C. Zeng and X. J. J. o. A. P. Zhang (2015). Bacillus amyloliquefaciens T1 as a potential control agent for cyanobacteria. **27(3)**: 1213-1221.

26 Guo, Z. and Y. W. J. J. o. A. P. Tong (2014). The interactions between Chlorella vulgaris and algal symbiotic bacteria under photoautotrophic and photoheterotrophic conditions. **26(3):** 1483-1492.

27 Dakhama, A., J. De la Noüe and M. J. J. o. A. P. Lavoie (1993). Isolation and identification of antialgal substances produced byPseudomonas aeruginosa. **5**(**3**): 297-306.

28 Gouveia, L., V. Veloso, A. Reis, H. Fernandes, J. Novais and J. J. B. T. Empis (1996). Evolution of pigment composition in Chlorella vulgaris. **57(2):** 157-163.

29 Lau, S., N. Shao, R. Bock, G. Jürgens and I. J. T. i. p. s. De Smet (2009). Auxin signaling in algal lineages: fact or myth? **14**(**4**): 182-188.

30 Mohite, B. J. J. o. s. s. and p. nutrition (2013). Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. **13**(**3**): 638-649.

31 Piotrowska-Niczyporuk, A. and A. J. P. G. R. Bajguz (2014). The effect of natural and synthetic auxins on the growth, metabolite content and antioxidant response of green alga Chlorella vulgaris (Trebouxiophyceae). **73**(1): 57-66.

32 Yu, Q., P. Li, B. Li, C. Zhang, C. Zhang and Y. J. J. o. B. M. Ge (2022). Effects of algal–bacterial ratio on the growth and cadmium accumulation of Chlorella salina–Bacillus subtilis consortia. **62**(**3-4**): 518-529.

33 Griffiths, M. J. and S. T. J. J. o. a. p. Harrison (2009). Lipid productivity as a key characteristic for choosing algal species for biodiesel production. **21**(**5**): 493-507.

34 Hempel, N., I. Petrick and F. J. J. o. a. p. Behrendt (2012). Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. **24**(**6**): 1407-1418