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PHYCOREMEDIATION OF OLIVE OIL WASTES USING CYANOBACTERIA FOR SUSTAINABLE BIOFERTILIZER AND BIODIESEL PRODUCTION

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ABSTRACT

The dual role of cyanobacteria in wastewater phycoremediation for sustainable biomass production combined with biorefinery approach is a feasible option. Phycoremediation is the process of employing algae for removing excess nutrient load from wastewater and subsequently diminish the pollution load. Industrial processes for olive production generate a considerable amount of oil wastewater, designated "olive mill wastewater" (OMW) known as *alpechin*, it caused serious environmental problems particularly in the Mediterranean areas where it is generated in huge quantities in short periods of time. The objective of this research was to study the ability of three cyanobacteria strains (*Nostoc muscorum*, *Anabaena oryzae* and *Spirulina platensis*) to grow, either individually or in a mixture, on relatively high olive mill wastewater (OMW) concentrations of 50, 75 and 100%. The highest phenolic compounds biodegradability and maximum biomass production have been taken as main criteria in the selection of the best treatment in this study. Best results of all growth parameters and phenolic compounds degradation were obtained by mixed culture and 50% OMW and these parameters make the potential of bio-formulating such these wastes into sources for olive trees bio-organic fertilizer is the most preferable methods for the agro-sustainable system. The cultivated algal species are suggested to be a promising feedstock for biodiesel, food and animal feed production according to the biochemical composition.

Keywords: cyanobacteria, phycoremediation, sustainable, biorefinery, OMW, biodiesel.

INTRODUCTION

Olive (*Oleaeuropaea* L.) is an evergreen tree belongs to family *Oleaceae*. It is a widely distributed tree grown in many arid zones of the world, native to all the countries around the Mediterranean region. Olive cultivation plays an important role in the economy of many countries, comparatively it resists drought and salinity conditions to a great extent in additions, it increases the land values where the soil is unsuitable for other crops, also countries to soil conservation. It helps to combat problems of environment and its protection that are currently of concern to nation authorities and organization (Sansoucy, 1984). This has prompted several to take up olive cultivation a commercial scale. The fruit of olive is of a major agricultural impotence in the Mediterranean region as a source of olive oil (Tous and Romero, 1994). Industrial processes for olive oil production generate a considerable amount of oil wastewater, designated "olive mill wastewater" (OMW) known as *alpechin* collected during the late fall and winter season. It is a significant by-product of olive mill industry caused serious environmental problems particularly in the Mediterranean areas where it is generated in huge quantities in short periods of time (Roig *et al*., 2006). The disposal of this waste in rivers decreases the dissolved oxygen content but increase the organic matter and K, Fe, Zn, and Mn contents (Yesilada *et al*., 1999).

Due to its high carbon and nitrogen contents, OMW has the potential to increase organic matter contents of soils (contains more than 94% organic matter). It can be used as a soil conditioner/ fertilizers amendment and proposed as one of the most suitable methods to restore soil fertility and resolve the problem of their disposal (Abu-Zreig and Al-Widyan, 2002). Even though the direct application of olive mill wastes on soil can be an approach of recycling nutrients and organic matter, the continuous application of OMW to soil without any treatment might cause unfavorable impact on plants (Kavdir and Killi 2008), soil microbial population and activity (Paredes *et al*., 1986 and1987; Kachouri and Hamdi 2004; Komilis *et al*., 2005), aquatic ecosystems (Della Greca *et al*., 2001) and even in air media (Rana *et al*., 2003) because of phenolic, fatty acid and mineral salts contents and high COD and BOD (Boubaker and Ridha, 2007; Kavdir and Killi, 2008). Phenolics are a group of dangerous toxic organic pollutants that are toxic to all the living organisms even at lower concentrations (Pimentel *et al*., 2008). At higher concentrations, it is very difficult to remove them from the environment, even by using physical and chemical techniques (Araña *et al*., 2001).

Water pollution became a global concern as wastewater loads at any point is now overweighed. Wastewater treatment is a central issue which has to be considered more seriously for the betterment of society and save water for our future. A well-understanding of the wastewater nature is essential in the proper design and operation of treatment stages (Kennedy KJ and Lentz EM, 2000; Metcalf E and Eddy H., 1991). Sustainable phycoremediation and biological treatment of different quality waters are suggested to be the main aspect of wastewater management and environment protection in a cheapest manner. Phycoremediation of OMW is a technology that use marine or cyanobacteria in the pollutants removal from wastewaters and CO_2 in biology

transform, and biomass propagation for biofuel industry (Mulbry W. *et al.,* 2008; Moreno-Garrido I., 2008; Olguın EJ *et al.,* 2004; Olguın EJ, 2003). This technology was investigated by John (John J., 2000) to refer to applied via algae growing. The first application was reported by Oswald (Oswald WJ and Gotaas HB, 1957) over 40 years ago for domestic wastewater treatment which is the research subject today (Oswald WJ., 1963; Oswald WJ., 1988). Extensive work has been achieved in recent years to test the algae sustainability in nutrients removal especially N_2 and P from wastewater effluents discharged into rivers or lakes (Hameed MSA., 2007; Aslan S., 2006; Hernandez JP *et al.,* 2006; Lebeau T and Robert JM., 2003; Mallick N., 2002). This study is successed in nutrients removal from OMW in a controlled manner, therefore environment deterioration is avoided.

Micro-Algae have already been found to detoxify OMW (Duarte *et al.*, 2011). In addition, EI-Sheekh *et al.* (2012) explored the ability of cyanobacteria to degrade phenolic compounds either by reduction, oxidation or by induction of some enzymes that degrade these toxic compounds. Cyanobacteria, as biofertilizer, are known to possess the ability to form associations with vascular/non-vascular plants and produce growth-promoting substances (Nanjappan-Karthikeyan *et al.,* 2007). They also known to increase soil fertility by enhancing the available N and P levels and exhibited an economical view that it can compensate about 50% of the recommended doses of N, P and K (Mahmoud *et al.*, 2015).

Therefore, the objective of this study is to develop the bioremedition technology of liquid wastes from olive oil production into valuable products via microalgae cultivation and formulate liquid bio-organic fertilizer to ensure the mitigation of chemical fertilizers usage in the field of olive cultivation and to reduce their potential adverse impacts on the environment and the economy. In addition to highlight the optimum bioreactors design for biomass production and its utilization in biofuel industry.

MATERIALS AND METHODS

Materials

Olive mill wastewater

Olive mill wastewater (OMW) was obtained from FIFA farm (km48 of Cairo-Alexandria, Egypt). The raw OMW samples were generated by the three-phase olive-oil extraction process of 2014 and 2015 seasons.

Samples were left in tanks to settle for 10 days under laboratory conditions (Markou *et al.*, 2010). The light supernatant after the sedimentation was filtered by passing through a sieve (mesh size about 200 µm) and was kept to be used as substrate for cyanobacteria cultivation. OMW chemical properties for the two seasons before and after settling are illustrated in Table-1.

Table-1. Chemical analysis of OMW during 2014 and 2015 seasons.

Season	pH	EC ds.m^{-1}	Organic Matter (%)	Organic carbon (%)	COD (%)	BOD_5 (%)	Total phenols (g/l)	N%	P%	K%	Na%	C/N ratio
Before sedimentation												
2014	5.2	5.71	21.2	12.6	-	-	-	1.62	0.68	1.14	-	84.92
2015	4.82	5.33	20.42	11.6	-	-	-	1.18	0.78	1.46	-	87.94
Average	5.01	5.52	20.81	12.1	-	-	-	1.4	0.73	1.3	-	86.43
After sedimentation												
2014	5.4	17.00	14.11	8.18	10.0	3.50	8.56	1.2	0.70	0.47	3.00	6.81
2015	6.75	15.75	12.75	7.40	6.67	2.65	7.70	1.1	0. 40	0.36	2.88	6.73
Average	6.07	16.4	13.43	7.79	8.34	3.08	8.13	1.15	0.55	0.415	2.94	6.77

Cyanobacteria strains

Three cyanobacterial strains i.e., *Nostoc muscorum*, *Anabaena oryzae* and *Spirulina platensis* were kindly supplied from Department of Microbiology; Soils, Water and Environment Research Institute (SWERI); Agricultural Research Center (ARC), Giza, Egypt. N_2-fixing *Nostoc muscorum* and *Anabaena oryzae* were maintained separately on BG_{11} medium (Rippka *et al.*, 1979). While, the non N_2-fixing *Spirulina platensis* was grown on Zarrouk medium (Zarrouk, 1966). Cultures were incubated in growth chamber under continuous shaking (150 rpm) and illumination (2000 lux) at 27± 2°C for 30 days to be used as inoculum for lab experiments.

Chemicals and reagents

Methanol (99.8% purity) as a reacting alcohol and concentrated sulfuric acid (H_2SO_4 conc.) as a biodiesel transesterification catalyst are supplied from El-Nasr Pharmaceutical Chemicals Company (ADWIC), Egypt.

Methods

Phycoremediation process

Procedure

Cyanobacteria culture suspensions at log phase, either individually or in a mixture of 1:1:1 v/v, were inoculated at the rate of 20% ($V_{inoculum}/V_{media}$) into 500 ml

conical flasks containing 200 ml of different sterilized dilutions of OMW/tap-water (25, 50 and 100% v/v). The non-inoculated OMW dilutions were used as control. In case of *Spirulina platensis*, all dilutions were supplemented with 5 g/l $NaHCO_3$ (Markou *et al.,* 2012). The cultures were incubated at 27±2°C under continuous shaking (150 rpm) and illumination (2000 Lux) for two weeks. Algal biomass development was determined as dry weight (Vonshak and Richmond, 1988). Culture characterization i.e., pH, Optical density at 560 (nm), total chlorophyll (mgl^{-1}) and algal cells dry weight ($g\ l^{-1}$) of the cyanobacteria for OMW inoculation at log phase were measured according to APHA (1998) and listed in Table-2.

Table-2. Characterization of studied cyanobacteria cultures.

Parameter	*Nostoc muscorum*	*Anabaena oryzae*	*Spirulina platensis*
pH	8.2	6.70	10.45
Optical density at 560 (nm)	1. 2	0.84	2.85
Total chlorophyll ($mg\ l^{-1}$)	5.39	5.31	13.50
Dry weight ($g\ l^{-1}$)	0.85	0.55	1.84

Up-scaling production of Cyano-OMW biofertilizer for field application

About 50-liter plastic tanks were used to prepare Cyano-OMW biofertilizer by diluting 20L of non-sterilized OMW with 20L tap water (1:1 v/v). Tanks were under continuous aeration by air pumps for two days before cyanobacterial inoculation. 10 L of cyanobacterial mixed culture at log phase (1:1:1 v/v) were added to 40 L of OMW-tap water diluted (1:1) and incubated for two weeks under laboratory conditions and continuous aeration to be applied for one season in the field.

Chemical Constituents of OMW and Cyano-OMW biofertilizer

Electric conductivity (EC) and pH values of OMW were measured after cyanobacteria cultivation period by Hach HQ40 (Vonshak and Richmond, 1988). Phenolic compounds were extracted according to the method of Elena De Marco *et al*. (2006) and were determined spectrophotometrically as described by Swain *et al*. (1959). Biochemical oxygen demand (BOD_5) was determined as the method described by Lenore *et al.* (1992). Chemical oxygen demand (COD), organic matter (OM), Dry Matter (DM) and organic carbon (OC) were measured according to APHA (1998).

Biodiesel production methodology

System set up

The cultivated cyanobacteria in OMW will be utilized as feedstock for biofuel synthesis based on their chemical composition. It is proposed to convert the algal lipids into fatty acid methyl esters (FAMEs) or biodiesel using direct transesterification process. The acid catalyst is firstly dissolved in the reacting alcohol and then fed to the biomass where in-situ transesterification reaction takes place. At the process end, the residual cake is removed and distinct layers of biofuel and glycerol are generated. Refining of products is achieved before storage.

Analytical method

Fatty acids composition of the produced *Spirulina* biodiesel was determined using gas liquid chromatographic analysis (GC) of the oil methyl esters. The chromatographic analysis was made using Hewlett Packard Model 6890 Chromatograph. A capillary column 30 m length and 530 µm inner diameter, packed with Apiezon® was used. Detector temperature, injection temperature and the column temperature were 280 ºC, 300 ºC and 100 to 240 ºC at 15 ºC/min, respectively.

RESULTS AND DISCUSSIONS

Phycoremediation process

Cultivating cyanobacteria on OMW for Cyano-OMW biofertilizer production

OMW under this study was characterized by its extremely high content of organic compounds, which is reflected by the high values of BOD_5, COD, OM, OC and total phenols as illustrated previously in Table-1. The BOD_5/COD ratio values of 0.35 to 0.40 were recorded at the first and second seasons, respectively which indicated the presence of poor biodegradable organic compounds and/or toxic ones (Adams *et al.*, 1997). Total organic matter concentrations ranged from 12.75 to $14.11 g.l^{-1}$ during the two seasons most of which are colloidal in nature making its settle ability very difficult. Application of biological treatment required diluting the OMW in order to keep the concentration of toxic compounds at a biologically tolerable level for cyanobacteria cultivation (Jaouani *et al.*, 2005). On the other hand, mineral analysis of OMW showed its rich in macro nutrients (NPK) that required for algal growth development. The obtained results of cultivating three cyanobacteria strains, either individually or in combination, on different dilutions of OMW indicated that all the biological inoculation affected significantly the chemical characteristics of OMW after being exposed to algal growth development for 2 weeks as shown in Table-3.

The acidic pH is a fundamental characteristic of mill olive effluent; pH values ranged between 4.0 and 5.6

for control treatments in all OMW dilutions. Cyanobacteria inoculation increased significantly pH values than those of control treatment. The highest mean pH values were recorded by cyanobacterial mixed culture and *Spirulina platensis* (7.93 and 8.17, respectively). However, *Anabaena oryzae and Nostoc muscorum* had no significant effect on pH values comparing with control. Regarding OMW dilutions, 50 and 100% concentrations increased pH values than 25%. These results are confirmed with the values obtained by Eroglu *et al.* (2008). It was noticed that the acidity was higher in all treatments after cyanobacteria growth reaching its maximum value of 8.5 by *Spirulina platensis* with 100% OMW. These results agreed with that reported by Ismail *et al.* (2013). This might be due to the formation of $CO_2/H_2CO_3/HCO_3^{-1}/CO_3^{-2}$ system which is a very useful buffer system for maintaining the alkaline pH which helps to prevent carbon depletion (Keskinkan *et al.*, 2012). Also, the increase of pH is may be due to the degradation of some phenolic acids present in the waste. The level of the pH reached at the end of the process values of 7.5 by cyanobacterial mixed culture with 50% OMW and 8.5 by *Spirulina platnsis* with 100% OMW those suitable for the cyanobacterial growth (Esmail *et al.*, 2014).

The electric conductivity (EC) measurement is a good assessment of the degree of mineralization of olive oil mill wastewaters, where each ion is characterized by its concentration and specific conductivity, the electrical conductivity is strongly related to the concentration of dissolved substances and to their nature (Di Serio *et l.*, 2008). In the case of olive oil mill wastewaters, the conductivity varies from one organism to another and it takes values ranging between 1.45 and 10.50 dsm^{-1}. All treatments significantly reduced EC values than control. The lowest EC values of 1.45 and 1.65dsm^{-1} were recorded by cyanobacterial mixed culture with 50 and 25% OMW, respectively as illustrated in Table 3. The important of EC as control parameter, agreed with that expressed by Oswald (1988), on the fact that total dissolved salt one among the decision factors that define the relationship culture medium species in the cultivation of microalgae. The reduction of EC values after cyanobacteria cultivation could be due to the ability of algal species to consume the nutrients during the algal growth (Mostafa *et al.*, 2012; Shanab *et al.*, 2012).

Table 3. OMW specifications after microalgae growing for two weeks.

Treatment	OMW %	pH	E.C ds m^{-1}	D.M g l^{-1}	COD g l^{-1}	O.C g l^{-1}	O.M g l^{-1}	Total phenols g l^{-1}
Un-inoculated	25	4.00	4.20	17.21	7.00	2.63	4.53	2.75
	50	4.90	6.00	23.14	10.00	3.75	6.47	4.38
	100	5.60	10.50	25.22	17.16	6.43	11.09	7.38
Mean value		*4.83*	*6.90*	*21.86*	*11.4*	*4.27*	*7.36*	*4.84*
Anabaena oryzae	25	4.30	1.60	18.72	2.18	0.82	1.41	1.20
	50	5.3	2.40	25.82	5.80	2.18	3.75	1.35
	100	4.50	5.00	26.11	9.20	3.45	5.95	3.75
Mean value		*4.70*	*3.00*	*23.55*	*5.73*	*2.15*	*3.70*	*1.99*
Nostoc muscorum	25	4.20	2.30	19.23	3.00	1.13	1.94	1.21
	50	5.20	3.00	26.05	4.65	1.75	3.01	1.75
	100	4.50	4.65	25.67	7.94	2.98	5.13	3.06
Mean value		*4.63*	*3.32*	*23.65*	*5.20*	*1.95*	*3.36*	*1.95*
Spirulina platensis	25	8.00	2.50	20.87	2.04	0.86	1.49	0.41
	50	8.00	3.25	27.27	3.66	1.37	2.37	1.90
	100	8.50	4.50	26.02	4.20	1.58	2.72	3.30
Mean value		*8.17*	*3.42*	*24.72*	*3.30*	*1.27*	*2.19*	*1.87*
Mixed Cyanobacteria	25	8.30	1.65	20.28	1.40	0.53	0.91	0.40
	50	7.50	1.45	28.32	2.20	0.82	1.42	0.27
	100	8.00	4.90	26.81	2.60	0.97	1.68	3.20
Mean value		*7.93*	*2.67*	*25.14*	*2.07*	*0.80*	*1.34*	*1.29*
OMW% Average	25	5.76	2.45	19.26	3.12	1.19	2.06	1.19
	50	6.18	3.22	26.12	5.26	1.97	3.40	1.89
	100	6.22	5.91	25.97	8.22	3.08	5.31	4.14

E.C.= Electric Conductivity; D.M= Dry Matter; COD= Chemical Oxygen Demand; O.C.= Organic Carbon; O.M.= Organic Matter

Yield, as an expression of organic production, is usually given in terms of dry matter of the organic mass produced over a period of time per unit volume (Tadros, 1988). Dry matter in OMW increased significantly by all cyanobacteria inoculation over the un-inoculated treatment (control) in response to algal biomass development. The highest dry matter value of 28.32 g/l was recorded by cyanobacterial mixed culture with 50% OMW as shown in Table 3. The intensive growth of algae was due to the high availability of nutrients (Jail *et al.*, 2010). Results of this study showed that cyanobacterial mixed culture had the highest biomass concentration of 5.2 g/l in 50% OMW dilution and all results are demonstrated in Figure-1.

OMW is characterized by its black brownish color that is considered undesirable property. Another negative impact of this wastewater is its extremely high content of organic compounds, which is reflected by the high values of COD and TOC, most of which are refractory or toxic to micro-organisms (Duarte, *et. al.,* 2012). However, the highest mean concentrations of COD, OC, OM and total phenols of the un-inoculated treatment (control) was significantly reduced from 11.14, 4.27, 7.36 and 4.84 g l^{-1}, respectively to 2.07, 0.8, 1.34 and 1.29 g l^{-1}, respectively in response to cyanobacterial mixed culture cultivation (Table-3). The biodegradation of phenolic compounds, COD and EC reflected fluctuation trends depended on either OMW dilution level or to the behavior of each cyanobacteria strain (Mostafa *et al.,* 2012; Shanab *et al.*, 2012). Cyanobacterial mixed culture recorded the highest reduction of phenolic compounds, COD and EC (93.8, 84.8 and 75.8%, respectively) with 50, 100 and 50% OMW, respectively after 2 weeks of incubation. These removal percentages were compatible with changing levels of the OMW concentration from 25-100% and led to increase the pollutant removal as reported by Ahmadi *et al*. (2006). In this respect, Mc Hugh (2003) reported that the uses of these microorganisms led to a progressive reduction of COD and BOD to values below the disposal limits.

Bioremediation mediated by microalgae and cyanobacteria is a viable new alternative for the detoxification of distillery-wastewaters, primarily due to the fact that they possess the advantage of a trophic independence for nitrogen and carbon. However, these are generally light dependent reactions that will necessitate a dilution of the colored effluents to be treated, in order to avoid light-blockade (Amores-Sanchez *et al.*, 2015). Although, OMW is difficult to treat by common biological processes due to high COD, low pH and the presence of compounds with phytotoxic and antibacterial effects (Sayadi *et al.*, 2000). *Anabaena oryzae, Nostoc muscorum and Spirulina platensis* individually were less efficient in degrading phenols, COD, OM, OC and EC at all OMW concentrations than cyanobacterial mixed culture which gave the best results. These results were agreed with those of Subashchandrabose *et al.* (2013) who found that the consortia of microorganisms can be efficient in detoxification of organic and inorganic pollutants and removal of nutrients from wastewaters compared to the individual microorganisms. They also reported that mixotrophy in cyanobacteria and microalgae can provide many competitive advantages over bacteria and fungi in degrading organic pollutants.

As mentioned before, sedimentation (or settling) operation was applied for 10 days and helped in removing suspended particles in OMW. In fact, most of the organic matter is in suspended forms. So, this process allows reducing COD by separating the supernatant with a low COD of a high COD settled sludge. Physical treatments are applicable to cleanse at least as a pre-treatment to OMW. Dilution is an efficient, cheap and simple process to reduce the organic load. Furthermore, filtration through a sieve (mesh size about 200µm) to allow particles in the range of 0.2 to 2 micrometers to pass. However, dilution increased the volume of effluent to treat, which results on bigger needs of storage and, consequently, associated economical costs (Duarte, *et. al.,* 2012). But in our study, dilution maximized the economic value of OMW waste instead of being disposed into the environment without any treatments. It was thus maximized through physical and biological processes that will eventually lead to a new cheap, sustainable and eco-friendly biofertilizer or biofuel like biodiesel.

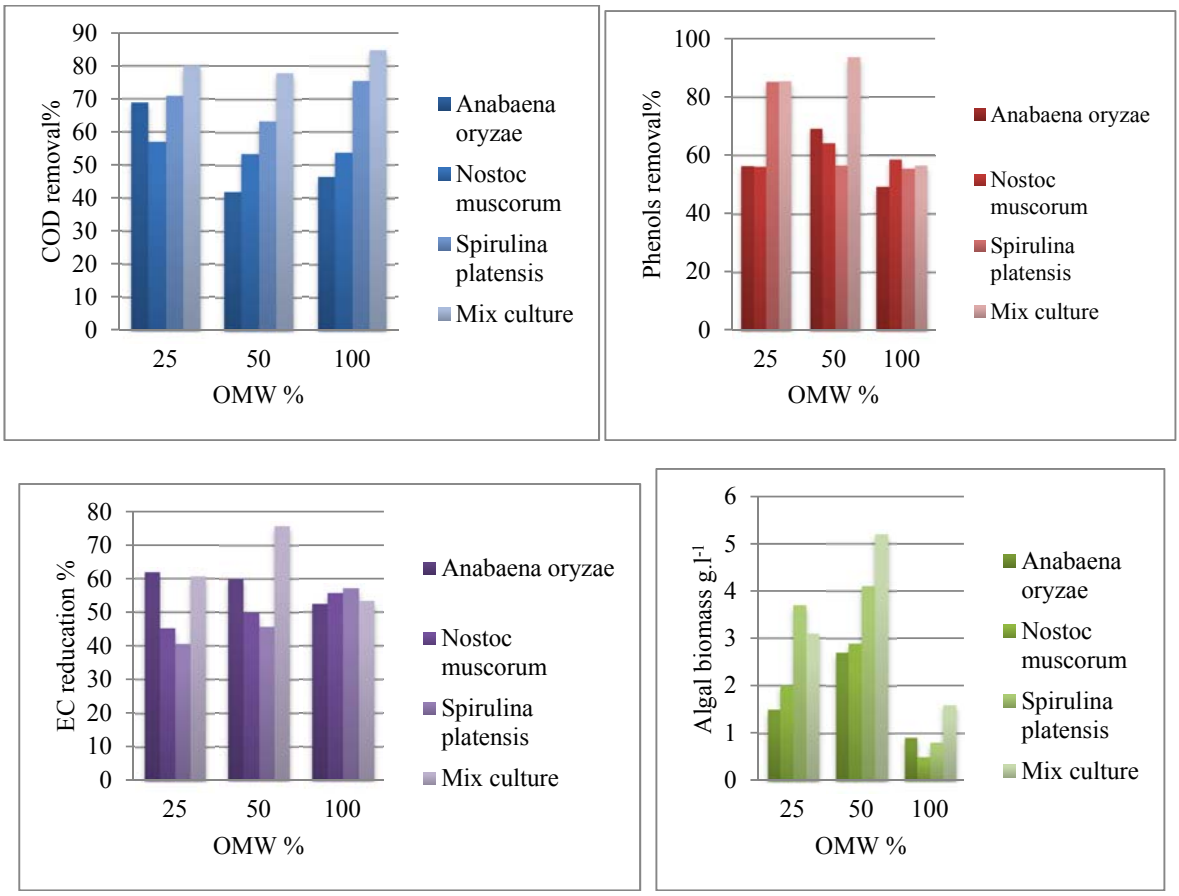

Figure-1. Phycoremediation results.

OMW of 50% concentration inoculated with cyanobacterial mixture was selected to be used as biofertilizer (Cyano-OMW) on large scale. In this concern, a strategy of multiple successive processes (sedimentation, filtration, aeration and bioremediation) was applied for Cyano-OMW large scale production. The physiochemical and microbiological properties of the bio-formulated Cyano-OMW are listed in Table-4. It proves that the product is neutral (pH 7.5 - 8.5) with moderate EC (2.5 - 3.75), very rich in macro and micro nutrients as well as free from pathogens.

Use of OMW as liquid fertilizer, due to its content in organic carbon, P, N and K, is often considered as a viable approach that restores soil fertility provided that land application is controlled and the soil type is appropriate and not associated with sensitive aquifers (Defra, 2009). Few studies that discussed the long term effects of OMW application on various types of agricultural soils and the fate of organic and inorganic contaminants in environmental media are available (Sánchez-Arias *et al.*, 2008; Ben Sassi *et al.*, 2006). Comparative effects of soil drench and foliar spray application methods of Cyano-OMW biofertilizer on soil properties, vegetative parameters, fruiting and yield of olive trees as well as the economic evaluation were investigated in 2015 by (Elbana, T.A. *et al.,* 2013).

Table-4. Specifications of bio-formulated Cyano-OMW.

Property	**Cyano-OMW**
Color	Brownish-green
Odor	-
pH	7.50 - 8.50
EC ($dScm^{-1}$)	2.50 – 3.75
Total solids (gl^{-1})	25.00 – 30.00
Chemical Oxygen Demand, COD (gl^{-1})	15.00 – 20.00
Organic matter (%)	2.50 – 3.00
Organic carbon (%)	0.88 – 1.20
Total nitrogen (%)	2.60 – 3.50
Total phosphorus (%)	1.70 – 2.00
Total potassium (%)	2.00 – 3.00
Phenols (%)	0.25– 0.30
Soluble nutrients ($mg\ l^{-1}$)	
N	1550.0 – 2000.0
P	1000.0 – 1500.0
K	1500.0 – 2000.0
Ca	1500.0 – 1700.0
Fe	50.0 – 60.0
Mn	5.0 – 6.0
Zn	2.2 – 2.5
B	10.0 – 11.5
Microbiological constituents of biofertilizer	
Total bacteria $X10^7$ CFU/ml	30.0 – 40.0
Coliform group	*ND
Faecal coliform	*ND
E.coli	*ND
Sallmonela and Shigella	*ND

*ND: Not Detected

Bioreactor design for phycoremediation of OMW

Effective phycoremediation of industrial wastewater like OMW and excessive algal biomass production require good design for controlled growth system. Open raceway ponds, photobioreactors, open oxidation ponds or vertical tank reactors are available models as bioreactors for simultaneous propagation of algal biomass and nutrients removal from OMW (Chinnasamy S. *et al.*, 2010; Munoz R. and Guieysse B., 2008). Successful bioreactor design for wastewater purification using cyanobacteria is an ongoing exercise (Briens C. *et al*., 2008).

There are not many options for large scale algae farms since algae needs sunlight, nutrients, and carbon dioxide in order to thrive and reproduce. The most primitive algae farmers used large open raceways of liquid medium to grow their algae as shown in Figure-2. Most raceways are designed with a current that constantly circulates the algae so that all organisms receive enough sunlight. Carbon dioxide is also pumped into the water to keep the algae alive, and nutrients are frequently replenished to support maximum growth. The advantages to raceways are that they are inexpensive, can support a large population of algae, and can use effluent water and CO_2 emissions from local industrial plants. However, open raceways are vulnerable to contamination and it is difficult to find an efficient way to harvest the algae from these large raceways due to the vast area and the constant water flow (I. Rawat *et al*., 2011). A common feature of most of the algal species produced commercially (i.e.

Chlorella, Spirulina and Dunaliella) is that they can be grown in open ponds systems and still remain relatively free of contamination by other microorganisms.

Egypt receives large amounts of daily solar radiation and has abundant flat desert land with large supplies of saline groundwater, sewage and pretreated industrial wastewater, making it an excellent location for algae growth. Two limitations of the site were the low nightly temperatures, which resulted in low productive species identified, and the climate change. Rectangular shape is the common used for open bioreactors where the width is in range of 4-6m and its length is 5-6 times of pond width. The depth is between 0.25m and 0.45m, and the distance between each two ponds is about 0.5m as illustrated in Figure-2 (Chisti Y. 2007).

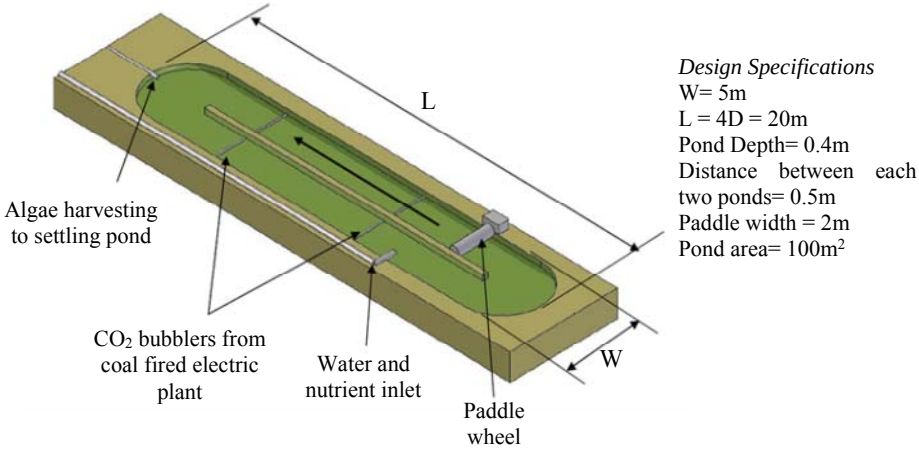

Figure-2. Scaled model of a140m^2 algae open pond bioreactor.

Cyanobacteria can be grown in vertical plastic sheets or photobioreactors (PBRs). These systems are flexible and its design can be optimized according to the features of the cultivated algal species. PBRs allow growth of many algal strains and provide a protected environment against contamination. PBRs benefits include lower CO_2 losses, lower water use and higher lipids productivity as can be predicted from Figure-3. In addition, biomass harvesting from PBRs is easy; as excess culture is overflowed. For proper design, a 0.15-0.25m is recommended for PBR diameter (D) and its length (L) is approximately 3-5 times the diameter (i.e. L/D = 3-5). Space between each two PBRs rows is about 1m espcially if they are inclined. Upto now, phycoremediation technology is not appropirate for a large scale industrial wastewater purification due to the absence of an optimum bioreactor design (Chisti Y., 2007).

Figure-3. Model of PBR investigated in this study.

Biomass production from OMW-Cyanobacteria suspension

In the past, microalgae have been produced as stage for wastewater purification and used as a food or feed supplement. Unicellular algae have efficiently proliferated in the nutrients uptake from wastewater effluents and this technology has been applied since 1950s (I. Rawat *et al.,* 2011). Phycoremediation of wastewater using algae is still occurring on small scale in the form of waste stabilization open ponds. In recent years, algae are assumed to be sustainable feedstock for alternate biofuels production. Therefore, it has been suggested that algae cultivation in wastewaters should concentrated on industrial scale lipids productivity (89). Previous investigations have shown the lipid productivity up to 505 mg L^{-1} day^{-1} and 30%wt. of algae biomass produced via

phycoremediation of wastewater (Wang L. *et al.,* 2010). Hence, the potential of algal biofuels production is currently feasible from economic point of view (El Shimi, H. *et al.,* 2016).

Effective phycoremediation process of wastewater using microalgae species for green fuels commercialization requires biomass recovery from the culture medium. This is considered an expensive part of industrial production of biomass that may contribute to 20-30% of the total production cost (L. Brennan and P. Owende, 2010). Selection of harvesting method for biomass production from OMW-cyanobacteria suspension is greatly important to the algal biofuels economics, and depends upon the characteristics of culture grown and algae specifications such as cell size and strain density (P. Schenk, 2008).

The optimal treated water suspension for industrial conversion is containing at least 300-400 g dry algae per liter, but algae that are feasible for OMW phycoremediation and biofuel production tend to be of low density, therefore economics of biomass harvesting seem to be difficult (I. Rawat *et al.,* 2011). Filtration, centrifugation, sedimentation, flocculation and flotation are all used for biomass recovery from OMW-cyanobacteria suspension (Mutanda T. *et al.,* 2010). For the case study, continuous centrifugation is preferred; as it is rapid and efficient, but not economical for industrial scale harvesting as it is highly energy intensive (Wang L. *et al.,* 2010). Sedimentation and filtration in conjugation with flocculation may be more feasible. Flocculation followed by centrifugation is the most suggested harvesting method for large scale biomass production from OMW suspension as shown in Figure-4.

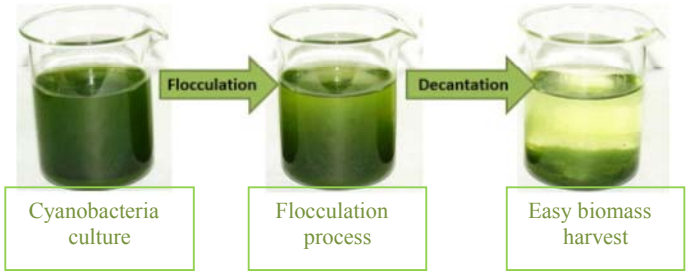

Figure 4. Mechanism of algae flocculation operation in settling pond

In particular, algae collection from offshores of the Mediterranean and Red Seas contain thousands of different algae strains, presents a good initial point for the development of microalgae biodiesel production system based on wastewater phycoremediation; as combination of these strains provide advances in the engineering genetic. So, algal strain selection and its chemical composition play viable roles in microalgae industries. Lipidomics, genomics, proteomics and metabolomics are future methodologies to develop new algal strains that exhibit high lipid productivity and feasibility to extract more valuable co-products (I. Rawat *et al.,* 2011).

Utilization of cultivated algae in biofuel industry

Once phycoremediation process has completed, the three microalgae strains are collected and dried followed by chemical analysis; to decide which strain can be considered a promising feedstock for biofuel (biodiesel or bioethanol) production. The biochemical analysis of algal biomasses is demonstrated in Table 5. As well known, the richest the lipids content algae the most suitable for biodiesel synthesis (El Shimi, H.I. *et al.,* 2013), so *spirulina* biomass is suggested a raw material biodiesel production, while the other cyanobacteria are preferred as bioethanol feedstock‘s due to their high carbohydrates content.

Table-5. Biochemical analysis of algal species investigated in phycoremediation process.

Compound	*Spirulina platensis*	*Nostoc muscorum*	*Anabaena oryzae*
	Dry basis %wt		
Lipids	11.05	9.6	10.3
Proteins	62.2	43.2	51.6
Carbohydrates	12.8	38.1	32.2
Minerals	6.95	5.5	4
Fibers	4	2.7	1.1
Nucleic acid	2.5	-	-
Moisture	0.5	0.4	0.8

The produced *Spirulina-platensis* was investigated for biodiesel production using the in-situ transesterification technology (R. Halim, *et al.,* 2012; B. D. Wahlen *et al.,* 2011; K. J. Harrington and C. D'Arcy-Evans, 1985). It involves simultaneous addition of alcohol and acid catalyst to biomass; as alcohol extracts the lipids and then catalyzed by the acid into fatty acid alkyl esters (FAAEs). This methodology is suggested to be economical way in algal lipids transformation into methyl esters (biodiesel) without pre-extracted oilgae (El Shimi, H.I. *et al.,* 2016). The feedstock "*Spirulina* microalgae" is dried at 110ºC; to evaporate the moisture content. Catalyst-alcohol solution is prepared and then added carefully to the dried biomass in a transesterification reactor. The former is produced by dissolution of H_2SO_4 catalyst (100% *wt./wt. oil*) into 80ml methyl alcohol on a magnetic stirrer for 5 min and freshly to maintain the catalyst activity. The reactor is adjusted at 65ºC for 8h. At the end of reaction, the warm mixture was allowed to cool in 15 minutes. Cascade filtration through filter-papers is achieved and the algal cake is washed at least two times by re-suspension in alcohol. The liquid mixture is transferred to a separating funnel and water was added; to facilitate the decantation of reaction mixture (biodiesel and glycerol). Further extraction of biodiesel was achieved by extracting three times using hexane, which generated two layers in 12 hours: hydrophobic layer (hexane, FAME and glycerides), and hydrophilic layer (water, glycerol, catalyst and excess methanol). Schematic diagram for algal biodiesel synthesis processes is presented in Figure-5. Once the products have fully settled, biodiesel layer on top looked thin, clear and deeply green in color, and glycerol layer at bottom looked thick and of light color. Hexane is removed by flash evaporation, while excess alcohol is distilled to purify the algal biodiesel. On the other side, glycerol was distilled to recover the alcohol residual then the former is dissolved in pure water for clarification. Neutralization of glycerin with NaOH is achieved; to eliminate the H_2SO_4 catalyst. The salt is decanted and glycerol is washed at least two times. Finally, glycerol is subjected to vacuum distillation for purification, and stored in a tank (El Shimi, H.I. *et al.,* 2013).

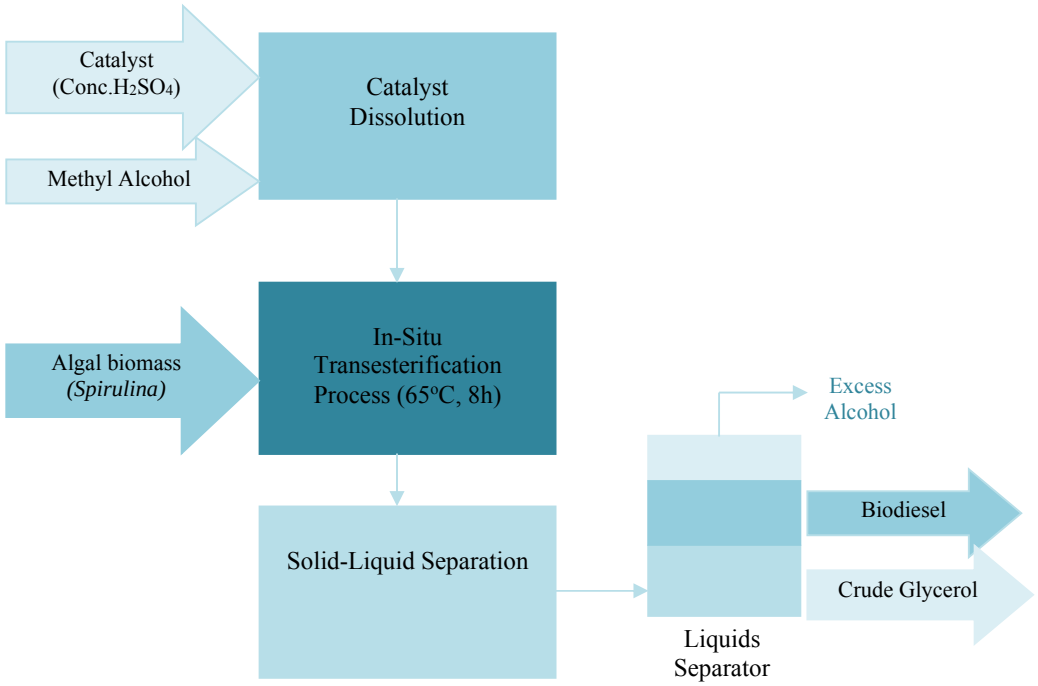

Figure-5. Proposed diagram for biodiesel production from *Spirulina.*

Fatty acid profile of the produced biodiesel produced was detected by GC-MS that confirming the predominant fatty acid groups in the *Spirulina-platensis* microalgae are Mystic, C14:0 (22.67% by mol) and Palmitic, C16:0 (49.58% by mol) as shown in Table 6, therefore *Spirulina* is a promising feedstock for biofuel synthesis. Also, algal cake is analyzed to optimize its utilization trend. The biochemical analysis was demonstrated for *S.platensis* cake and proved presence of valuable amount of protein (51.5%wt), hence it is suitable to be animal fodder or poultry diet feed. Considerable amount of nitrogen (500 mg/100g), phosphor (900 mg/100g) and potassium (1475 mg/100g) are involved in *S. platensis*. These elements are the main nutrients for plant growth, so it is vital as promising plant nutrition element to be used as N-P-K fertilizer "bio-fertilizer"

Table-6. Analysis of cyanobacterial processing products.

FAME Profile		Analysis of Algal Cake		
Fatty acid	***Spirulina* Biodiesel**	**Component**	**Value**	**Unit**
Mystic methyl ester (C14:0)	22.67	Carbohydrates	12.6	%wt
Palmitic methyl ester (C16:0)	49.58	Protein	51.5	%wt
Palmitoleic methyl ester (C16:1)	2.75	Moisture	1.5	%wt
Stearic methyl ester (C18:0)	5.56	Ash	7.5	%wt
Oleic methyl ester (C18:1)	2.24	Ca	400	mg/100g algae
Linoleic methyl ester (C18:2)	5.03	P	900	mg/100g algae
Linoleuic methyl ester (C18:3)	7.41	Fe	70	mg/100g algae
Ecosanoic methyl ester (C20:0)	1.06	N_2	500	mg/100g algae
Eicosenoic methyl ester (C20:1)	3.69	K	1475	mg/100g algae
Saturated	78.87			
Monounsaturated	8.68			
Polyunsaturated	12.44			

CONCLUDING REMARKS

Phycoremediation is successor in nutrients removal from OMW especially phenols using 50% OMW and Mix Culture of the three cyanobacteria strains although its bioreactor design is an ongoing exercise however; open ponds or PBRs are feasible. Biomass collection can be achieved by flocculation followed by centrifugation to obtain about 300g dry algae/liter. Phycoremediation produced microalgae suitable for biodiesel as C16:0 is the predominant fatty acid in lipids profile. Residual algalcake can be utilized as solid biofertilizer or animal fodder.

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