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EFFECT OF MUTAGENS ON BIOMASS AND LIPID CONTENT OF *ULOTHRIX* SP. AND *OSCILLATORIA* SP. FOR IMPROVED BIODIESEL PRODUCTION

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ABSTRACT

Chemical and physical mutagens like sodium azide, H_2O_2 and UV light have a significant role in the metabolism of living organisms. Effect of these mutagens was recorded during the present work on lipid content of *Oscillatoria* sp. and *Ulothrix* sp. The algal species were subjected to the mutagens by adding them in culture media. Lipids were extracted by Microwave- assisted extraction using methanol, ethanol and chloroform. During the present work generally a significant effect of algal species and mutagens was observed. All the tested mutagen significantly affected the lipid content. A significant effect of solvent on lipid content of algal species was observed when the algae were extracted with different solvents after treatment with H_2O_2 and UV light however this effect was non-significant when algae were subjected to sodium azide. . In general, the mutagen resulted in an increase in lipid content. In *Oscillatoria* sp. the lipid content was generally less as compared to Ulothrix sp. The highest lipid content of 0.5433±0.002 $g100g^{-1}$ was recorded in *Ulothrix* sp. after 3 days stress of sodium azide stress followed by H_2O_2 treated (50mM) samples with a content of 0.5133±0.05$g100g^{-1}$. In *Oscillatoria* sp. 15mM treatment for 3 days gave a lipid content of 0.3733±0.0001$g100g^{-1}$ after 3 days. A reduction in fresh weights of both tested algal species was recorded after UV stress. The oil content also showed a significant reduction after UV exposure. GC-MS analysis of the transesterified lipids showed that different fatty acids show variation when algal samples were treated with mutagens. Hence this study is helpful in manipulation of algal oil for biodiesel production.

Key Words: Mutagen, Oscillatoria, Transesterification, Ulothrix
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INTRODUCTION

The necessity of energy is increasing day by day, due to the increasing industrialization, development and population. This energy is obtained from different sources such as petrol, nuclear power and natural fuel like oil, coal and gas. The main drawback of utilizing fuels based on petroleum is that they contribute to the environmental pollution. The burning of petroleum emits greenhouse gases that contribute to environmental pollution and thus causes global warming. Production of petroleum, particularly refining, produces toxic materials, such as carbon monoxide and plastic. So if we choose petroleum as a main energy source, our ecosystem will degrade very soon (Hossain *et al.*, 2008).

Microalgae are capable of reproducing on their own by the help of photosynthesis, they need sunlight and nutrients to grow. They can grow nearly everywhere if supplied with definite nutrients and adequate aeration (Aslan and Kapdan, 2006; Pratoomyot *et al.*, 2005; Renaud *et al.*, 1999). Researchers has now focused on microalgae to produce biodiesel because of high amount of biodiesel production from vegetable oils. The content of lipid in microalgae can be increased by manipulating the conditions of culture environment (Rodolfi *et al.*, 2009). Some chemicals as Sodium azide and Hydrogen peroxide may act as a mutagen to have a change in metabolism. During the process of photorespiration, hydrogen peroxide forms in the cells (Grant and Loake, 2000). Hydrogen peroxide is a powerful oxidizing agent, it first starts with the local damage by oxidation, then destroy the metabolic functions of the cell and in the end it destroys the whole cell (Mallick and Mohn, 2000). Ultraviolet light unpleasantly disturbs the microalgae. At similar strengths, ultraviolet-A is less damaging and limited to the cell as compared to ultraviolet-B (Pessoa, 2012). Ultraviolet-B has negative effect on the process of photosynthesis while ultraviolet-A may encourage photosynthesis. Under low ultraviolet-B, there is no significant increase in lipid content and in treatment with high ultraviolet-B lipid content per cell increases (Pessoa, 2012; Rastogi and Incharoensakdi, 2013; Xue *et al.*, 2005).

During the past few ages, biodiesel has gained much consideration as a renewable and approachable fuel because of shrinking petroleum reserves (Vasudevan and Briggs, 2008). Fatty acid alkyl ester is a biodiesel, resulting from the vegetable oils or lipids and alcohol with or without the existence of a catalyst, is an effective and alternate diesel fuel. The process used for converting vegetable or animal oils into biodiesel is called trans-esterification (Janaun and Ellis, 2010; Khola and Ghazala, 2012). It is better for environment, less toxic

and its use reduces our dependence on smuggled fuel and contributes to our own economy (Mata *et al.*, 2010). Various different forms of renewable biofuels can be provided by microalgae, and can produce energy in several different ways (Hossain *et al.*, 2008). Due to the high photosynthetic efficacy, biomass production and rapid growth rate microalgae are considered excellent for biofuel production. The accumulation of lipid in the cells of microalgae lies between 25–90% (dry weight). For the biodiesel production from microalgae the most important steps are sampling, identification, treatment with mutagens, harvesting and oil extraction, its trans-esterification and biodiesel analysis (Mubarak *et al.*, 2015).

In algal biotechnology, there is a need for algal strains with a potential for improved biodiesel production, present study involves the mutagenic effect of three chemical mutagens on the selected algal strains to check their effect on lipid production. Hence it will help in utilization of algal species for the enhanced biofuel production.

MATERIALS AND METHODS

Sampling and Identification: Samples of microalgae were collected from different places of Lahore (Lahore Zoo, LCWU, Punjab University and Jallani Park). Identification of these isolates was made by the morphology of the individual cells following microscopic examination.

Preparation of Nutrient Media and Treatment with Mutagens: BB (Bold's basal) culture media was prepared for the cultivation of algae strains. The pH was maintained within the range 6.8 – 7.0 for the efficient growth. Each algal culture was treated with different concentrations of sodium azide (control, 5mM and 15mM), hydrogen peroxide (control, 20mM and 50mM)and and UV light (control, 30 sec and 60 sec) and then cultivated in the culture media at optimum pH (6.8), light and temperature of 38 and 31 ºC for *Ulothrix* sp. and *Oscillatoria* sp. respectively as reported earlier (Lee *et al.*, 2014; Sharif *et al.,* 2015). Samples were harvested at day 3 and 6 for biomass and lipid response.

Harvesting, Oil Extraction and Transesterification: After cultivation the biomass was harvested and air dried (Hossain *et al.*, 2008). Dried algal biomass was grounded with motor and pestle. Oil was extracted with methanol, chloroform and ethanol using Microwave assisted extraction (Hossain *et al.*, 2008).

Catalyst and methanol mixture was transferred in an eppendorf containing algal oil. The biodiesel was separated from sedimentation by centrifugation. Biodiesel obtained was stored for further analysis (Hossain *et al.*, 2008). Biodiesel was analyzed GCMS analysis.

Statistical Analysis: The data were analyzed using Univariate analysis of variance (SPSS Version 25) at ($P \leq 0.05$). The data were analyzed by two way ANOVA using 4 sources of variation (Algae species, treatments, solvents and time period/days. Standard deviation of the mean values was calculated for each treatment. F test was applied to the data to analyze the data for significant differences.

RESULTS AND DISCUSSION

The collected algal samples were examined on the basis of their structural characters as well as the cellular characters using microscope. The morphological comparison with other microalgal strains identified two strains *Ulothrix* sp. (filamentous green algae) and *Oscillatoria* sp. (cyanobacteria).

Lipid extraction by MAE: Microwave assisted technique was used for the extraction of lipids from dried algal biomass as this method is considered best as compared to others as the technique is quiet cheap, harmless and quick (Mandal *et al.* 2007).

Effect of Sodium Azide on oil content of Different Algal species: The effect of sodium azide on lipid content of tested algal species is given in Table 1. No physical change was observed after 3 and 6 days of sodium azide stress. It was observed that after 3 days stress of sodium azide on *Ulothrix* sp. the extracted lipids were 0.33±0.00g100g^{-1}, 0.3233±0.01 g100g^{-1} and 0.3600±0.001 g100g^{-1} with methanol, ethanol and chloroform solvent respectively. It is depicted in Table 4 that significant difference was recorded in the oil of different algal species. Likewise the concentration of mutagen has a significant effect on the oil content. However, the solvent was found to have non-significant effect on the lipid content. The oil extracted from *Oscillatoria* sp. was 0.1700±0.002 g100g^{-1}, 0.2533±0.002 g100g^{-1} and 0.2800±0.04 g100g^{-1} after 3 days. Highest oil concentration was recorded when both the algal species were subjected to 15mM stress of Sodium azide for 6 days and extraction with methanol solvent 0.5100±0.002 g100g^{-1} for *Ulothrix* sp and 0.3733±0.0001 g100g^{-1} for *Oscillaoria* sp.

Sodium azide is a chemical mutagen which can affect the different constituents (Kodym and Afza, 2003. The results of the present study are in line with the findings of Al- Qurainy (2009) who reported that at all concentrations of sodium azide may change the metabolism of the algae.

Effect of Hydrogen Peroxide on oil content of Different Algal species: In hydrogen peroxide treatment there was a slight difference in the color. The significant effect of effect of two different concentrations of hydrogen peroxide 20 mM and 50 mM on lipid content

was observed after 3 and 6 days, then compared it to the control (Table 2). It is evident from Table 4 that hydrogen peroxide stress significantly increased the lipid content. After 3 days stress of 500 mM stress of hydrogen peroxide on *Ulothrix* sp. the amount of lipid obtained was 0.5433±.05 g100g^{-1}, 0.3267±.01 and 0.3800±.02 with methanol, ethanol and chloroform solvent. It was observed that the weight of algae showed reduction in the weight of algae but the oil content showed increased. This may be due to the effect of stress conditions on metabolites of algae. . In *Oscillatoria* sp. The samples treated with 500uL H_2O_2 had 0.3733±.01 g100g^{-1}, 0.3433±.05 g100g^{-1} and 0.3433±.01 g100g^{-1} oil content when extracted with different solvents. As hydrogen peroxide is a strong oxidizing agent it inhibits the growth of microalgae but increases the lipid content. At the concentration of 50mM greater amount of lipids was found than 20 mM. Similar kind of results were found in the study of Drabkova *et al.* (2007) and Barrington and Ghadouani (2008) which showed that by using different amounts such as 2 mM, 4 mM and 6 mM, H_2O_2 had deadly effect on cells of algae and displayed growth inhibition with the increase in lipid productivity.

Table 1: Effect of sodium azide stress on lipid content of *Ulothrix* sp. and *Oscillatoria* sp.

Algal strain	Concentration	Stress Duration	Lipid g100g^{-1}			
			Wet biomass (g)	Methanol Solvent	Ethanol Solvent	Chloroform Solvent
Ulothrix sp.	Control	3 days	2.533±0.01	0.3333±0.001	0.3233±0.01	0.3600±0.001
		6 days	2.0±0.01	0.3133±0.002	0.3300±0.01	0.3700±0.001
	5mM	3 days	1.5±0.01	0.4273±0.003	0.3200±0.02	0.3700±0.003
		6 days	2.3±0.01	0.4390±0.004	0.3367±0.002	0.3900±0.001
	15mM	3 days	2.6±0.01	0.5433±0.002	0.3267±0.01	0.3800±0.002
		6 days	2.1±0.01	0.5100±0.002	0.3733±0.02	0.4200±0.02
Oscillatoria sp.	Control	3 days	1.98±0.02	0.1700±0.002	0.2533±0.002	0.2800±0.04
		6 days	1.96±0.03	0.1833±0.002	0.2700±0.02	0.2800±0.02
	5mM	3 days	2.2±0.01	0.2323±0.002	0.2600±0.02	0.2900±0.02
		6 days	2.2±0.02	0.2367±0.002	0.2757±0.02	0.3300±0.02
	15mM	3 days	2.0±0.01	0.3067±0.002	0.2667±0.02	0.3000±0.02
		6 days	1.99±0.01	0.3733±0.001	0.3433±0.02	0.3433±0.02

Table 2: Effect of hydrogen peroxide stress on lipid content of *Ulothrix* sp. and *Oscillatoria* sp.

Algal strain	Concentration	Stress Duration	Biomass	Lipid g100g^{-1})		
				Methanol Solvent	Ethanol Solvent	Chloroform Solvent
Ulothrix sp.	Control	3 days	2.533±0.01	0.3333±0.001	0.3233±0.01	0.3600±0.001
		6 days	2.0±0.01	0.3133±0.002	0.3300±0.01	0.3700±0.001
	20 mM	3 days	2.00±.01	0.2923±0.01	0.3200±0.01	0.3667±0.01
		6 days	1.56667±.06	0.4390±0.08	0.3367±0.05	0.3900±0.01
	50 mM	3 days	2.50±.01	0.5133±0.05	0.3267±0.01	0.3800±0.02
		6 days	2.73±.017	0.5100±0.01	0.3733±0.01	0.4200±0.01
Oscillatoria sp.	Control	3 days	2.1±.01	0.1700±0.02	0.2533±0.05	0.2767±0.05
		6 days	1.98±.01	0.1833±0.02	0.2700±0.01	0.2800±0.01
	20 mM	3 days	1.96±.01	0.2323±0.08	0.2600±0.02	0.2900±0.01
		6 days	2.20±.01	0.3133±0.01	0.2757±0.01	0.3300±0.01
	50mM	3 days	2.0±.01	0.3067±0.04	0.2667±0.01	0.3000±0.01
		6 days	1.99±.00	0.3733±0.01	0.3433±0.05	0.3433±0.01

*Wet biomass (g) and lipids (g) represented in table are the mean of 3 replicates ±S.D

Effect of UV light on oil content of Different Algal species: The effect of UV light on the two algal species is given in Table 3. Both algal species significantly differ in their oil content. Exposure to UV light stress resulted in an increase in lipid content in *Ulothrix* sp. after 30 sec stress lipids obtained 0.3840±0.05 g100g^{-1}, 0.3200±0.0

$g100g^{-1}$ and 0.3800±0.01 $g100g^{-1}$ for methanol, ethanol and chloroform solvent. In Ulothrix sp. at day 3 sample extracted with methanol solvent 0.3467±0.01 $g100g^{-1}$ oil was found which showed reduction at day 6 in the samples treated with 60 sec UV radiation. Likewise, a reduction in the lipid content was recorded in the other samples exposed with UV radiation for 60 sec. with the lipid content of 0.3467±0.01 and 0.2167±0.01 $g100g^{-1}$ at day 3 and 6 in samples extracted with methanol. However, a deviation from this trend was recorded in the samples extracted with ethanol and methanol.

Table 3: Effect of UV light stress on lipid content of *Ulothrix* sp. and *Oscillatoria* sp.

Algal strain	Concentration	Stress Duration	Fresh Weight (g)	Lipid (%)		
				Methanol Solvent	Ethanol Solvent	Chloroform Solvent
Ulothrix sp.	Control	3 days	2.533±0.01	0.3333±0.001	0.3233±0.01	0.3600±0.001
		6 days	2.0±0.01	0.3133±0.02	0.3300±0.01	0.3700±0.001
	30 sec	3 days	1.55±0.03	0.3840±0.05	0.3200±0.00	0.3800±0.01
		6 days	1.40±0.01	0.3767±0.06	0.3367±0.01	0.3900±0.02
	60 sec	3 days	0.87±0.04	0.3467±0.01	0.2600±0.05	0.2800±0.00
		6 days	0.70±0.02	0.2167±0.01	0.2767±0.02	0.3267±0.02
Oscillatoria sp.	Control	3 days	1.97±0.02	0.2367±0.08	0.2533±0.01	0.2733±0.02
		6 days	1.97±0.02	0.2633±0.08	0.3100±0.05	0.3167±0.05
	30 sec	3 days	1.30±0.02	0.2423±0.02	0.2567±0.01	0.3233±0.05
		6 days	1.37±0.05	0.2467±0.05	0.2757±0.09	0.3000±0.06
	60 sec	3 days	0.60±0.02	0.3067±0.04	0.2667±0.01	0.2667±0.07
		6 days	0.73±0.02	0.2400±0.03	0.2433±0.04	0.2300±0.02

Table 4: Analysis of variance for the effect of different factors on lipis after treatment of algae with Sodium Azide, Hydrogen Peroxide and UV Radiation.

		Sodium Azide		Hydrogen Peroxide		UV Radiation	
Source of Variation	DF	MS	P values*	MS	P values*	MS	P values*
Algae	1	0.301	0.000	0.171	0.000	0.115	0.000
Conc.	2	0.046	0.000	0.098	0.000	0.025	0.000
Solvent	2	0.003	0.394	0.013	0.027	0.008	0.038
Days	1	0.000	0.815	0.058	0.000	0.002	0.377
algae * conc	2	0.051	0.000	0.004	0.307	0.011	0.010
algae * solvent	2	0.010	0.033	0.023	0.003	0.004	0.198
algae * days	1	0.012	0.045	0.001	0.552	0.002	0.377
conc * solvent	4	0.085	0.000	0.018	0.001	0.002	0.500
conc * days	2	0.007	0.091	0.000	0.882	0.004	0.170
solvent * days	2	0.020	0.001	2.269E-5	0.994	0.008	0.026
algae * conc * solvent	4	0.026	0.000	0.003	0.544	0.003	0.316
algae * conc * days	2	0.009	0.050	0.007	0.135	0.006	0.075
algae * solvent * days	2	0.023	0.001	0.001	0.719	0.005	0.128
conc * solvent * days	4	0.011	0.007	0.006	0.151	0.002	0.367
algae * conc * solvent * days	4	0.010	0.014	0.002	0.589	0.001	0.885
Error	72	0.003		0.004		0.002	
Corrected Total	107						

DF = Degrees of freedom; MS = Mean square. *Significance level $\alpha = 0.05$.

In *Oscillatoria* sp. more drastic reduction in biomass was recorded after UV stress. The sample treated with UV radiation for 60 sec had a fresh weight of .73000±0.02 g as compared to control (2.0±0.01 g). The oil content in the algal samples was also reduced after 6 days indicating that UV radiation exposure might have damaged the cells. The sample at day 3 had oil content of 0.3067±0.04 $g100g^{-1}$, 0.2667±0.01 $g100g^{-1}$ and 0.2667±0.07 $g100g^{-1}$ which reduced to 0.2400±0.03

$g100g^{-1}$, 0.2433±0.04 $g100g^{-1}$ and 0.2300±0.02 $g100g^{-1}$ respectively.

UV light stress of 30 secs influence the lipid content of both *Oscillatoria* sp. and *Ulothrix* sp. but not to the greater extent. While 60 sec exposure showed reduction in the lipid content that further results into lower production of biodiesel. Skerratt *et al.* (1998) observed the effect of UV light on lipid content of three antarctic marine phytoplankton and found that the lipid content per cell decreases under high UV light treatment. Thus it was observed that the lipid content was significantly affected by the concentration of mutagen and type of solvent (Table 4).

Transesterification of lipids to biodiesel and GC-MS Analysis: After transesterification the lipids were converted into biodiesel. Prepared biodiesel was further analyzed by using GC-MS (Table 5). For *Ulothrix* sp. the maximum percentage of octanoic acid found was 15.3% after 6 days of 15mM sodium azide stress at RT 8.9. The amount of octadecadienoic acid was 12.5% and octadecenoic acid was 33.6% at RT 16.3 and 16.5 respectively. Under hydrogen peroxide stress of 50mM concentration 11.9% octadecanoic acid, 15.8% hexadecanoic acid, 19.7% hexadecenoic acid and 28.7% octadecenoic acid was found for *Oscillatoria* sp.

Table 5: Relative percentage of compounds detected in biodiesel by GC-MS

Sr. No.	Compound	RT	*Ulothrix* sp. subjected to NaN_3 stress (15mM)	*Oscillatoria* sp. subjected to H_2O_2 stress (500µl)	*Ulothrix* sp. subjected to UV stress	*Oscillatoria* sp. subjected to UV stress
1.	Octadecanoic acid	6.0	4.7	2.6	2.1	2.4
2.	Decanoic acid	6.4	2.7	0.4	-	0.8
3.	Propanoic acid	7.2	-	-	0.9	5.8
4.	Pentanoic acid	7.8	10.1	2.7	1.8	9.5
5.	Octanoic acid	8.9	15.3	3.4	4.3	3.1
6.	Hexadecanoic acid	13.26	4.9	15.8	4.1	12
7.	Tetradecanoic acid	13.95	6.9	6.6	10.4	15.3
8.	Octadecadienoic acid	16.3	12.5	8.2	4.6	11
9.	Octadecenoic acid	16.5	33.6	28.7	65.4	34
10.	Octadecanoic acid	17.0	4.7	11.9	-	1.5
11.	Hexadecenoic acid	20.3	4.6	19.7	6.4	4.6

For UV stress of 2 min 10.4% tertradecanoic acid was found at RT 13.95 for *Ulothrix sp.* while 65.4% octadecenoic acid was found due to the random mutation of UV light. For *Oscillatoria* sp. 15.3% teteradecanoic acid and 34% octadecenoic acid was present at RT 13.95 and 16.5 respectively.

Transesterification process converted the lipids into diesel. Base-catalyzed transesterification was done because it is faster than the acidic transesterification. According to Lotero *et al*. (2005), acid-catalyzed process is 4000 times slower as compared to base-catalyzed one. The process of transesterification for obtaining biodiesel from algae and analysis has been reported earlier (Mercer and Armenta, 2011) indicating the presence of fatty acids thus suggesting algal oil a suitable candidate for biodiesel production.

Conclusion: From the results of the present study it can be concluded that chemical mutagens significantly enhanced the lipid content of the tested algal strains. Hence treatment with sodium azide and hydrogen peroxide is an effective approach to enhance lipid content in *Oscillatoria* sp. and *Ulothrix* sp. which in turn resulted in an improved biodiesel production. On the other hand, UV light stress was found less effective for the lipid content. Hence lipid can be enhanced by treating the algae with sodium azide, hydrogen peroxide or UV radiation which eventually led to biodiesel production.

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