Study on sustainable and integrated approach for the generation of low-cost algal biomass and biofuel by using agro-industrial wastewater

SUMMARY
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Neha Gupta
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Prof. D.P. Singh

DEPARTMENT OF ENVIRONMENTAL SCIENCE
SCHOOL FOR ENVIRONMENTAL SCIENCES
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A Central University, NAAC Accredited 'A' Grade)
VIDYA VIHAR, RAEBARELI ROAD
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SUMMARY

Algae have developed broad tolerance to environmental stress conditions including high nutrient levels. This advantage has led to the wide use of the algae in bioremediation of wastes, resulting in treated waters as well as the production of useful biomass (El-Din, 2019; Olguin, 2003). The algal biomass serves as feedstock for several valuable products, including food, feed, fertiliser, pharmaceutical and biofuel production. Nutrient removal by algae is economical, sustainable, simple and beneficial for the environment (Filippino et al., 2015). Integration of wastewater treatment and biomass production may lead to economic savings as well as the opportunity to avoid costly wastewater treatment method and production of high-value algal biomass (Gupta et al., 2019). Microalgae have been found to be promising in the removal of nutrients primarily nitrogen and phosphorus, BOD, and COD (Aslan & Kapdan, 2006; Lebeau & Robert, 2003). Further, significant progress in the field of cultivation of microalgae coupled with treatment of wastewater has resulted in the improvement in the production of algal biomass and biofuel production (Salama et al., 2017).

The present investigation was an attempt to study the ability of wild-type and mutant strain of *Scenedesmus vacuolatus* to withstand the various environmental stresses. The overall stress tolerance ability was exploited for wastewater treatment, biomass production and improvement in the accumulation of lipid- a source of biofuel. The first objective of the work was to isolate a DCMU-tolerant mutant strain of microalga *S. vacuolatus*, which has the potential to sequester more carbon due to as a modification in the photosynthetic apparatus of the alga. The growth of microalgae under harsh environmental conditions such as limited nitrogen nutrition and high light intensity and different spectral quality of light was also optimized with respect to photosynthetic efficiency, biomass and lipid content. Further, agro-industrial wastewater was used as a nutrient source for growth, while remediation of wastewater was carried out to reduce the pollutant load.

The isolated algae from Leh, Ladakh, India was identified by 18S rRNA analysis as *Scenedesmus vacuolatus* (MH459062). Further, for the improvement of algal strain for production of higher biomass and lipid content was achieved by

improvement in the photosynthetic apparatus of *S. vacuolatus* by random chemical mutagenesis by using EMS (ethyl methanesulfonate) as a chemical mutagen. According to Larkum et al. (2012) and Stephens et al. (2010), mutagenesis is an effective tool to select mutant strains of microalgae, which are capable of synthesizing higher lipid content. The selection of mutant strain after mutagenesis of *S. vacuolatus* was done by screening the mutant population, using a sub-lethal concentration of DCMU (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea) a herbicide (also known as diuron, direx, karmex). DCMU is known to inhibit the photosynthetic electron transport by blocking the electron transfer at the level of QB site of photosystem II (Manandhar-Shrestha et al., 2009; Huber & Edwards, 1975). The comparative study of wild-type (WT) and mutant strain demonstrated that about 2 fold greater DCMU-tolerance in the mutant strain as compared to wild-type (WT).

The mutant strain was further evaluated in terms of its photosynthetic characteristics by measuring the fast chlorophyll fluorescence induction parameters (OJIP curve) and was compared with the WT. The results revealed that the photosynthetic performance of the mutant strain was better than the WT in the presence as well as the absence of DCMU. A higher degree of DCMU-tolerance in the mutant strain of *S. vacuolatus* might be associated with changes in the DCMU binding 33-kDa D1 protein and turn over in the photosynthetic apparatus (Matto et al., 2018; Astier et al., 1984; Erickson et al., 1984). Further, a high non-photochemical quenching (NPQ) value in the mutant strain than the WT might be the main reason behind high light intensity tolerance of the mutant strain as evident from the value of rETR (Relative Electron Transport Rate) measured under varying photon flux density.

Further, the analysis of the morphology of both WT and mutant strain by Scanning electron microscope (SEM) and flow cytometer, revealed that mutant strain has smaller cell size, in comparison to WT cells. However, optimum pH (7.8) and temperature (25℃) condition both the WT and mutant strain were the same. The analysis of lipid content by flow cytometer during different phases of growth of both WT and mutant strain showed higher lipid content during the stationary phase of both the strains when compared with exponential and lag phase of growth.

Another study based on the effect of light quality and light intensity on the photosynthetic efficiency and cells constituents of microalgae *S. vacuolatus*. The result showed that mutant strain was relatively more tolerant to high light stress (60 µmol m^{-2} s^{-1}) as compared to WT (40 µmol m^{-2} s^{-1}). The high light intensity between 80-100 µmol m^{-2} s^{-1} caused a drastic decrease in the growth, protein and carbohydrate content of both the strains. FTIR analysis of cells grown under varying light intensities (10-100 µmol m^{-2} s^{-1}) also showed changes in the protein, carbohydrate and lipid content of both the strains. FTIR and flow cytometric analysis of cell biomass demonstrated light intensity-dependent decrease in protein and carbohydrate, but lipid content was increased. However, the cell constituents including lipid was higher in the mutant strain (60 µmol m^{-2} s^{-1}) than the WT (40 µmol m^{-2} s^{-1}). Results on the photobleaching of pigments, lipid peroxidation, loss of -SH groups and RNO bleaching indicated a greater level of ROS generation in the WT than the mutant.

The photosynthetic parameters (F_v/F_m, ET_0/RC, TR_0/RC, ABS/RC, and RC/ABS) (Stirbet & Govindjee, 2012) studied indicated better photosynthetic performance and electron transport in the mutant strain than the WT even under the high light intensity. The quantum yield (F_v/F_m) and PI_{abs} or RC/ABS of the mutant strain, suggested better light utilization efficiency in the mutant strain than the WT. The captured photons in case of the mutant were found to be largely dissipated in terms of heat as evident from the higher value of NPQ. Whereas, a higher value of rETR with higher photosynthesis saturating light intensity in the mutant strain supported the hypothesis that mutant was metabolically well adapted to handle the excess photons due to its altered photosynthetic system, which supports better light tolerance and photosynthetic efficiency in the mutant than the WT.

Whereas the result of light quality demonstrated that WT cells are grown well under the green light, whereas mutant strain showed better growth under the orange light condition. There was little growth in both the strains under the blue light regime. FTIR analysis of WT and mutant strains confirmed the variable synthesis of cell constituents. The flow cytometric analysis of neutral lipids in both the strains revealed increased in the lipid of WT under green and red light, whereas mutant strains exhibited maximum lipid under orange and yellow light. Interestingly, a differential response of both WT and mutant strains exhibited changes in the cell constituents, including lipid, in response to varying spectral qualities of light as well as light intensity, strain-specific characteristics of the photosynthetic machinery.

The OJIP induction kinetics of chlorophyll fluorescence was used to evaluate the photosynthetic characteristics of the PSII reaction centre of both WT and mutant strain under the different spectral qualities of light. The overall quantum yield (QY) of the mutant strain was found to be higher than that of the WT, irrespective of the spectral quality of light irradiance used during the growth. The current results also revealed a higher value of non-photochemical quenching (NPQ) in the WT cells than the mutant, indicating greater dissipation of absorbed photo-energy by WT in the form of heat. The highest use efficiency of mutant strain was better than WT, resulting into its higher biomass and lipid contents.

Further study on the effect of varying nitrogen sources (sodium nitrate, ammonium chloride, sodium glutamate and different carbon: nitrogen (C/N) ratio) on the growth, biochemical constituents, lipid production in the wild-type (WT) and mutant strain of *S. vacuolatus* demonstrated nitrogen limiting condition was more effective in enhancing the lipid production but biomass content was reduced in both WT and mutant strain. Further, the result suggested that growth and biochemical constitutes in the mutant strain were better in the presence of all the nitrogen sources as compared to WT. However, the best growth of both WT and mutant strain was observed under the mixotrophic (carbon: nitrogen) and nitrate supplemented conditions but the lipid content was reduced. On the contrary lipid content was highest in the glutamate supplemented and nitrogen starved conditions. It is possible that the mode and type of nitrogen nutrition may influence carbohydrate and protein synthesis (De Farias et al., 2018; Chen et al., 2013). The nitrogen limiting condition is described to be one of the most important key regulators for triggering lipid synthesis in microalgal cells (Wu et al., 2013).

In the present investigation, glutamate was used as a nitrogen source to support growth and synthesis of biochemical constituents including lipid in both WT and mutant strain. Based on the results, reduced functioning of the nitrogen assimilatory system in the glutamate grown and nitrogen starved cells of both the strains was found to be the main reason for the higher accumulation of lipid as evident from the flow cytometry analysis in both the strains. The overall findings suggested that mutant strain showed better growth, biochemical constituents and lipid content as compared to WT.

The microalgal strains of *S. vacuolatus* were used for the treatment of soybean and poultry wastewaters. The results on both WT and mutant strain grown on the soybean and poultry wastewater were compared with bore-well water (tap water) and synthetic medium (BG-11). The results exhibited higher biomass and biochemical constituents in soybean and poultry wastewater grown cells of both WT and mutant strain, when compared with BG-11 and bore well (tap water) grown cells. However, microalgal growth and biochemical constituents in both the WT and mutant strain grown on poultry wastewater was maximum when compared with the corresponding value of both the strains grown on soybean wastewater. The tap water grown cells exhibited the lowest biomass and biochemical constituents. However, BG-11 grown cells of both the strain showed higher biomass and biochemical constituents than the tap water, but much lower than that shown by soybean and poultry wastewater. The result also indicated that utilisation of wastewater nutrients by the mutant strain was better than the WT.

FTIR analysis of microalgal biomass and lipid content of both WT and mutant cells were grown on tap water, BG-11, soybean and poultry wastewater showed that triglycerides (TGAs) were the dominant component of lipids in the mutant strain. Whereas lipoprotein and phospholipid were the major components of lipid in WT cells. The harvesting of algal biomass or settling of biomass is another hurdle in the mass cultivation of microalgae. The result of biomass settling and recovery efficiency (η) were found to be higher in WT (50-60%) than the mutant strain (35-40%) under varying temperature and pH conditions, perhaps, due to differences in the cell size of both the strains. The present investigation suggested that temperature can be one important factor to determine the settling ability of cells.

In the present investigation, soybean and poultry wastewaters grown microalgal biomass was used for biodiesel production and remediation of wastewater. The results demonstrated that nutrient load was efficiently reduced form soybean and poultry wastewaters when both the strains (WT and mutant strain) were grown at appropriate dilution of wastewater. The overall nutrient removal efficiency of both WT and mutant strain was in the range of 40-90% when grown exclusively on the soybean and poultry wastewater. However, removal of the nutrient load of soybean wastewater was faster than the poultry wastewater in the presence of both the strains.

Further LC-MS (liquid chromatography-mass spectrometry) analysis of microalgae *S. vacuolatus* of both the strains (WT and mutant strain) grown on soybean and poultry wastewater, BG-11 and tap water showed higher accumulation of carbohydrate by both strains grown on soybean wastewater when compared with that in the poultry wastewater grown cells. The mannose was the most predominant sugar followed by fructose, galactose and maltose in the presence of soybean and poultry wastewater of both strains. The result of amino acid accumulation demonstrated that soybean wastewater was a good source of nitrogen nutrition than the poultry wastewater, particularly for amino acid synthesis. The accumulation of amino acid in both the strains was promoted by soybean and poultry wastewater as compared to the BG-11 and tap water. Further, the results depicted that most of the essential fatty acid content was higher in both the WT and mutant strain when grown in the presence of poultry wastewater, followed by soybean wastewater, BG-11 and tap water. The overall results also revealed that unsaturated fatty acid content was higher in both the strains, mainly dominated by mainly linolenic acid (C18:3) and elaidic acid (C18:1). However, the overall level of lipid and fatty acid in the mutant strain was found to be better than the WT under different nutritional conditions.

Methyl esters of fatty acid were analysed in the biodiesel of soybean and poultry wastewater, BG-11 and tap water grown cells by using GC-MS. The higher biodiesel concentration was obtained from the poultry wastewater grown cells, followed by soybean wastewater, BG-11 and tap water grown cells. The results revealed the presence of monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and saturated fatty acid (SFA). The biodiesel from the microalgal irrespective of source of nutrition showed mainly methyl esters of hexanoate (C6:0), palmitic (C16:0), palmitoleate (C16:1), stearate (C18:0), linoleate (C18:2), linolenate (C18:3), behenate (C22:0), erucate (C22:1), lignocerate (C24:0), nervonate (C24:1) and docosahexaenoic (C22:6).

Hence, foregoing results revealed that the unique characteristics of the DCMU-tolerant mutant strain of *S. vacuolatus* was its higher light intensity tolerance, higher photosynthetic performance and better nutrient removal efficiency as compared to WT strain. The biomass productivity, lipid and biofuel production capability of the mutant strain was better than the WT. These attributes of DCMU-tolerant mutant strain make it an efficient tool to develop a sustainable, eco-friendly technology to integrate the phycoremediation of wastewater and biofuel production at a very reduced cost.

Flow chart showing characteristic features of wild-type and mutant strain of *S. vacuolatus*