ejbps, 2019, Volume 6, Issue 9, 144-154. **Research Article** **SJIF Impact Factor 4.918**

EUROPEAN JOURNAL OF BIOMEDICAL
AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 6
Issue: 9
144-154
Year: 2019

OPTIMIZING THE CRITICAL ENVIRONMENTAL FACTORS INFLUENCING THE GROWTH AND LIPID PRODUCTION OF MICROALGAE

Y. Neeraja*[1] and Prof. Ammani K.[2]

[1]Department of Microbiology, Ch.S.D.St. Theresa's Autonomous College for Women, Eluru.
[2]Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur.

***Corresponding Author: Y. Neeraja**
Department of Microbiology, Ch.S.D.St. Theresa's Autonomous College for Women, Eluru.

Article Received on 23/06/2019 **Article Revised on 13/07/2019** **Article Accepted on 03/08/2019**

ABSTRACT

Optimizing the critical environmental factors is essential for the development of mixed algal cultures for lipid production. Design of experimental (DOE) methodology using Taguchi orthogonal array (OA) was applied to evaluate the influence and specific function of eight important factors (Carbon(Glucose), Nitrates, Phosphates, Trace metal(Fe), pH,Salinity and Light) on lipid production. Three levels of factor variations were selected, which represent the experimentation size by symbolic arrays of matrix (L_{18}-18 experimental trails) with an OA layout of $2^1 \times 3^7$. All the eight factors were assigned with three levels except for nutrient stress operation (2^1). The most influential factor was light, accounting for 16.8% of the overall variance followed by carbon supplementation (12.8%) and nutrient stress (9.3%). pH, phosphates and trace metals showed zero influence on the production process with respect to variance. Altogether, light, carbon and nutrient stress contributed a majority of 38.9% at their individual levels on the total lipid production indicating that these factors played critical role in the optimization. The validation experiments performed with the obtained optimized conditions showed enhanced total lipid production of 31.9%.

KEYWORDS: Design of experimental (DOE), orthogonal array (OA).

INTRODUCTION

Rapid usage of fossil fuels is now recognized as highly unsustainable because of their limited supplies and contribution to atmospheric pollution. Fossil fuels are also regarded as the major source of greenhouse gases responsible for global warming (Leeuwen *et al.*, 2011). To overcome these consequences lot of attention is being directed towards the development and utilization of alternative energy resources (Leeuwen *et al.*, 2011). In this direction, biofuel production from photosynthetic microorganisms is being considered as one of the viable strategies to produce renewable energy **(**Ma FR and Hanna MA1999**,** Vicente *et al.*, 2004**)**. Due to their higher photosynthetic efficiency and lipid content, this can be converted into bioenergy such as biofuels (biodiesel, bioethanol and bioelectricity) and combustible gases (Fig 4.1).

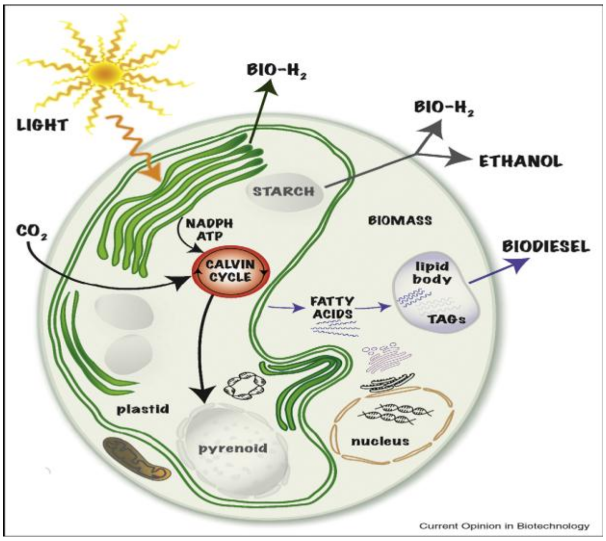

Fig 4.1: Metabolic pathways in green algae related to Biofuel and Biohydrogen production.

Biodiesel can be produced from a variety of raw material, including plant oils, animal fats and waste oils as well as microalgae (Vicente *et al.*, 2004**).** Each raw material has its advantages and disadvantages in terms of oil content, fatty acid composition, biomass yield and geographic distribution. Depending on the origin and quality of raw material, changes may be required for the production process of biodiesel. The use of plant oils as biodiesel raw material has been long recognized and well documented in numerous studies (Demirbas, 2008). These raw materials include the oils from soybean, rapeseed, palm, canola, peanut, cottonseed, sunflower and safflower. Based on the geographic distribution, soybean is the primary source for biodiesel in USA, palm oil is used as a significant biodiesel raw material in Malaysia and Indonesia, and rapeseed is the most common base oil used in Europe for biodiesel production **(**Vicente *et al.*, 2004**).**

The vast majority of these plants are also used for food and feed production. Thus, the use of these plant oils as raw material for biodiesel seems insignificant for the developing countries which are importers of edible oils (Abdullah *et al.*, 2009). In addition to these edible oils, various non-edible, tree-borne oils from jatropha, karanja, jojoba and neem are the potential for biodiesel raw material (Abdullah *et al.*, 2009**,** Meher *et al.*, 2008). Jatropha and karanja are two oilseed plants that are not widely exploited due to the presence of toxic components in the oils. In India, they are popularly used as biodiesel raw material.

In addition to the plant oils, animal fats and waste oils are the potential sources for commercial biodiesel production (Meher *et al.*, 2008). Among these raw material, tallow, lard, yellow grease and waste cooking oils have received most interest (Jain and Sharma, 2010). However, animal fats and waste oils usually contain large amounts of free fatty acids, which can be as high as 41.8% (Banerjee and Chakraborty, 2009). Free fatty acids cannot be directly converted to biodiesel in alkali-catalyzed transesterificaton but react with alkali to form soaps that inhibit the separation of biodiesel from glycerine and wash water fraction (Banerjee and Chakraborty, 2009). A two-step process was developed for these high fatty acid raw materials: acid-catalyzed pre-treatment and alkali-catalyzed transesterificaton. Because animal fats and waste oils have relatively high level of saturation (Banerjee and Chakraborty, 2009), the biodiesel from these sources exhibits poor cold flow properties.

Microalgae represent a wide variety of aquatic photosynthetic organisms with the potential of producing high biomass and accumulating high level of oil. The production of biodiesel from microalgal oil has long been recognized and been evaluated in response to the United States Department of Energy for research in alternative renewable energy (Canakci, 2007). Currently, the commercialization of algae-derived biodiesel is still in its infancy stage. Using microalgae as biodiesel raw material has received unprecedentedly increasing interest, including but not restricted to microalgal strain selection and genetic engineering, mass cultivation for biomass production, lipid extraction and analysis, transesterificaton technologies, fuel properties and engine tests (Sheehan *et al.*, 1998). Considering their unique characteristics, microalgae have been considered as the most promising raw material of biodiesel that has the potential to displace fossil diesel.

2. Experimental methodology

2.1 Biodiesel production from microalgae

2.1.1 Microalgae

Mixed microalgae culture collected from the Tammileru Stream (TH), and Kolleru lake (Composite Cultures) in Eluru city was used as biocatalyst. Sampling of microalgae was carried out in pre-monsoon season. The algal cultures were collected in polythene bags and transferred to the laboratory immediately. The cultures exhibited significant variations in their characteristics viz., appearance, occurrence, colour and texture. Prior to experimentation, the cultures were washed twice with water to remove epiphytes and sand particles.

The culture was washed twice with water and pelletized (2.1 g with a concentration of 0.2 g/l) by centrifugation (3000 rpm; 10 min at 30°C) to remove associated debris and restored in rectangular plastic tubs (36 cm × 24 cm × 12 cm) exposed to diffused sunlight. The culture tub was fed with Domestic sewage (DS; pH, 7.5; COD, 400 mg/l; TDS, 750 mg/l, nitrates, 115 mg/l) to provide carbon and nutrient source for microalgal cultivation. During the operation the temperature of the tubs was as 28 ± 3 °C. After 8 days of growth period till the consistent amount of biomass (cell density) was achieved. The cultures were harvested .The dewatered culture settled at the bottom was scrapped using thin aluminium sheets. The algal biomass was uniformly spread over a glass plate and allowed for drying under diffused sunlight. After drying, the algal-biomass(Culture) was finely powdered using a blender, weighed and then stored this was used as inoculum for further analysis.

2.1.2 Taguchi's DOE methodology

DOE methodology employing Taguchi's approach was used. The designed methodology can be broadly classified into four segments viz., Plan, Perform, Analyze and Validate. Each segment abides by well defined objectives, which are further interconnected in sequence to achieve the overall optimization of the processes (Kang *et al.*,2004, Venkata Mohan *et al.*,2005).

2.1.2.1 Experimental plan

The first segment facilitates selection/identification of important factors whose variation had a critical effect on the lipid production. Eight factors viz.,light, pH, temperature, nutrient stress, salinity, nitrogen, phosphorous and trace metals were selected for optimization due to their significant role in the synthesis of lipids (Table 1).

Table 3.3: Selected factors and assigned levels.

S.No	Factor	Level 1	Level 2	Level 3
1	Nutrient stress	Yes	No	
2	Carbon supplement (glucose)	0 g	5g	10g
3	Nitrates ($NaNO_3$)	0	200 mg	400 mg
4	Phosphates (mg/l)	0	200 mg	400 mg
5	Trace metal (Fe) (mg/l)	0	50 mg	100 mg
6	pH	6.5	7.5	8.5
7	Salinity (NaCl)	0 M	1.5 M	3 M
8	Light (4000 Lux)	24 h Light	24 h Dark	12/12

Subsequently, an experimental matrix was designed. Taguchi method facilitates to organize large number of experimental situations described as orthogonal array (OA) to reduce errors and to enhance the efficiency and reproducibility of the laboratory experiments (Li *et al.*, 2007). Three levels of factor variations were selected, which represent the experimentation size by symbolic arrays of matrix (L_{18}-18 experimental trails) with a OA layout of $2^1 \times 3^7$(Table 2). All the eight factors were assigned with three levels except for nutrient stress operation (2^1). In the designed OA, each column consisted of a number of conditions depending on the levels assigned to each factor and the diversity of factors can be studied by crossing OA of control factors.

Table 3.5: Orthogonal array (L18 ($2^1 \times 3^7$)) of designed experiments.

Experiment number	Factors								Lipid Productivity (% of dry biomass)
	1	2	3	4	5	6	7	8	
1	1	1	1	1	1	1	1	1	21.3
2	1	1	2	2	2	2	2	2	11.4
3	1	1	3	3	3	3	3	3	30.2
4	1	2	1	1	2	2	3	3	28.3
5	1	2	2	2	3	3	1	1	21.2
6	1	2	3	3	1	1	2	2	22.9
7	1	3	1	2	1	3	3	3	28.4
8	1	3	2	3	2	1	1	1	18.39
9	1	3	3	1	3	2	2	2	29.5
10	2	1	1	3	3	2	1	1	9.18
11	2	1	1	3	3	2	2	2	8.4
12	2	1	3	2	2	1	3	3	18.3
13	2	2	1	2	3	1	2	2	25.3
14	2	2	2	3	1	2	3	3	29.2
15	2	2	3	1	2	3	1	1	18.3
16	2	3	1	3	2	3	2	2	28.4
17	2	3	2	1	3	1	3	3	16.7
18	2	3	3	2	1	2	1	1	10.6

2.1.2.2 Data analysis

The data derived from the experiments was processed through a software (Qualitek-4; NutekInc) to analyze the output using 'bigger is better' performance characteristics (Table 3.3 and 3.4).

2.1.2.3 Validation

The optimized process conditions established from the proposed methodology (Table 3.5) were further validated for lipid production in batch experiments.

2.1.2.4 Analysis

Algal biomass growth was monitored by measuring OD at 600 nm, 650nm and changes in pigment concentration (chlorophyll *a* and *b*) was measured at 647 and 664 nm. Total soluble carbohydrate content (TSCC) in the algal cells was determined by Anthrone method. Bioprocess monitoring in terms of COD, pH, nitrates and phosphates were performed according to the standard methods outlined.

2.1.2.5 Major factors governing microalgae biomass production

The relative influence of the individual factors on biomass production can be summarized in descending order of Carbon >Nitrates > Phosphates > Trace metals >Light.

The maximum biomass was observed in 10 g/l glucose, dark condition (0.402 g/l), followed by 0.398 g/l in heterotrophic 10 g/l glucose, 12:12 (light:dark) condition and 0.382 g/l in 5 g/l glucose, heterotrophic dark conditions.

Carbon

Usually the availability of carbon source favors biomass growth as it is important for cell division and for carrying out the physiological activities in a cell. Carbon is considered as a key factor governing the growth pattern of microalgae in both photoautotrophic, mixotrophic and heterotrophic mechanisms. When glucose is given as carbon source, it is directly absorbed into the cell by simple diffusion via hexose/H+ symport system. This transport is more favourable easy and less energy consuming when compared to CO_2 uptake by photosynthesis. Also the conversion of CO_2 is complicated than glucose, as the glucose directly involves in glycolysis and increases the rate of metabolism. The increase in the concentration of glucose increases biomass because more energy is released in form of ATP and NADPH, when glucose is broken down. Among photoautotrophic and heterotrophic modes, heterotrophic cultivation of microalgae offers several advantages including minimization of light requirement, good control over cultivation process, higher growth and easy biomass harvesting (Bahadar and Khan, 2013).

Nitrates and phosphates

Nitrates and phosphates concentration of 400mg/l has shown high biomass production of 0.4 and 0.39 g/l. Nitrogen is a fundamental element for the formation of proteins and nucleic acids. It is usually assimilated in microalgae in the form of nitrates, nitrites and ammonia for synthesis of cellular growth proteins, are responsible for increase in biomass. Being an integral part of essential molecules such as ATP, the energy carrier in cells, phosphate is another very important nutrient. Phosphate is also a part of the backbone of DNA and RNA, which are the essential macromolecules for all living cells. It is an important component required for normal growth and development of algal cells (Islam *et al., 2003*). It has been shown that phosphorus, rather than nitrogen, is the primary limiting nutrient for microalgae in many natural environments.

Iron(Fe)

It was observed that trace metals like iron and potassium had significant influence on biomass growth (0.4 g/l and 0.398 g/l). Trace metals like iron and potassium are important for normal growth, photosynthesis and respiration in algae. Iron acts as redox catalyst in photosynthesis and nitrogen assimilation and mediates electron transport reactions in photosynthetic organisms (Goldman, 1973). Potassium helps in the ion transport channels and maintenance of osmotic potential within the cell.

Light

Light intensity affects growth of algae through its impact on photosynthesis (Stockenreiter *et al.*,2013). Although rate of growth under increasing light intensity is a function of strain and culture temperature, the growth rate of algae is maximal at saturation intensityand decreases with both increase or decrease in light intensity (Danquah *et al.*, 2009). Adaptation to different light intensities takes place through multiple mechanisms such as changes in types and quantities of pigments, growth rate, dark respiration rate or the availability of essential fatty acids 9246). Light intensity is also known to affect the cellular composition of algae. Low light intensity has been observed to result in higher protein content while high photon flux density (PFD) results in increased extracellular polysaccharide content (Sorokin and Krauss, 1958). In addition to total light intensity, light cycles and the spectral composition of incident light have an impact algal growth. In our study, maximum biomass (0.398 g/l) was obtained at 12:12h of day and night cycle.

RESULT AND DISCUSSION

3.1 Influence of individual factors on microalgae lipids production

The designed experimental conditions showed significant variations on performance. The selected parameters showed high degree of influence on biomass. The average effect of the factors along with interaction at the assigned levels on the performance of lipid production is depicted in table 3.6. The difference between the values at levels 2 and 1 (L2–L1) of each factor indicated the relative influence of the effect. The larger the difference, the stronger is the orthogonal array (L18 (21 x 37)) of designed experiments. The negative values were ignored in assessing the main effect, as the placement order of levels were assigned either positive or negative values. The relative influence of the factors on lipid productivity can be summarized in descending order of

Carbon>Salinity>Nitrates>Nutrientstress>Light>Phosphates>pH>Tracemetals.

CARBON

Among the selected factors, carbon supplementation showed highest influence on the total lipid productivity while trace elements, pH and phosphorous showed least influence. At level 1, no glucose was added that served as autotrophic condition. At level 2 and 3, glucose was supplied at 5 g/l and 10 g/l concentrations, respectively. At 10 g/l glucose concentration, maximum total lipid content of 30.4% and 29.5% DCW was achieved and 27.2% DCW at 5 g/l was achieved (**Fig 1**). Moreover highest neutral lipid of 12.3% was observed at 10 g/l and 10.3% at 5g/l glucose and dark conditions. Organic carbon in the form of glucose showed direct influence on total lipids and neutral lipid accumulation. Carbon when supplied as glucose gets converted into intermediates like pyruvate and acetyl coA and then towards fatty acid synthesis in plastids.

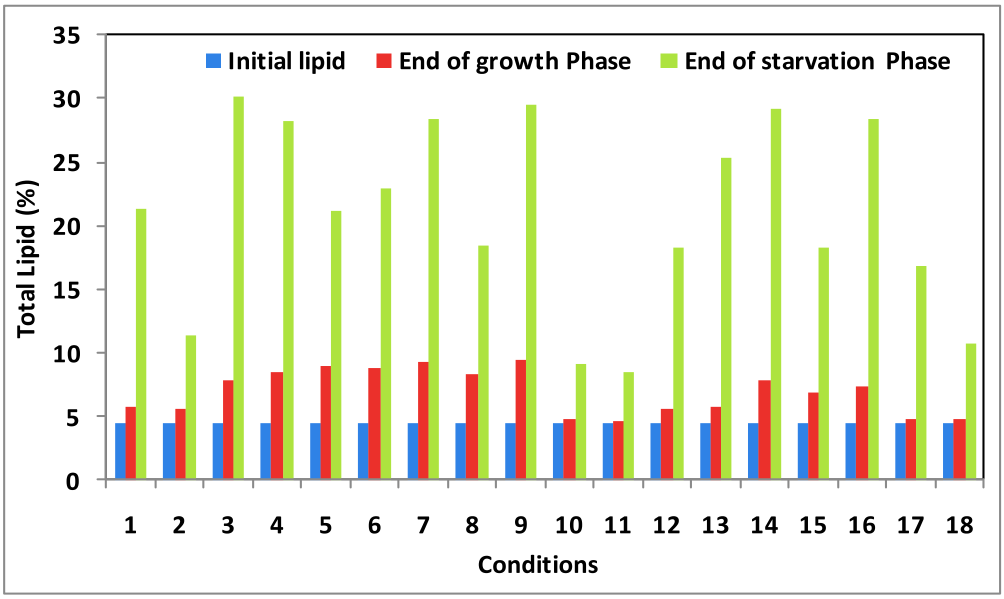

Fig 3.19 Lipid profiles of all the experimental conditions.

The presence or absence of carbon source determines the mode of nutrition to be autotrophic, mixotrophic or heterotrophic. Glucose, sucrose, and starch are the main carbohydrate products of algal photosynthesis. Under normal conditions, autotrophic algal cells accumulate few amounts of lipids, usually less than 10% of dry cell weight in microalgae **(**Islam *et al.*, 2003). In mixotrophic operation, both cell growth and biosynthesis of products are significantly influenced by both the nutrients present in the medium and by the environmental factors (Wältermann *et al.*, 2005**)**. The underlying mechanism might be interpreted by the negative relationship between photosynthetic starch accumulation and lipid synthesis. Li et al. (2010) observed that triacylglycerol (TAG) was over produced by 10-fold in a *Chlamydomonas* starchless mutant with inactivation of ADP glucose pyrophosphorylase.

This work suggests a strategy to increase lipid production by directing more photosynthetic carbon partitioning to lipid. But as genetic manipulations are involved with high cost inputs and low product formation, heterotrophic growth is more favorable for higher lipid synthesis.

The utilization of the substrate increases drastically when simpler and higher energy storing molecules are available such as glucose. The synthesis of TAG in algae was inclined towards supply of carbon in the growth medium. Initially algae utilize the available carbon source in the medium for growth and cell maintenance, then produces biomass to synthesize membrane lipids like phospholipids and glycolipids responsible for maintenance of cell wall structure and integrity. The remaining or excess carbon after cellular activities will be used for accumulation of TAG or storage lipids within the cell. Carbon forms as a structural backbone to most of the living things including microalgae in the form of macromolecules like carbohydrates, lipids and protein molecules. Thus carbon capture and utilization by the microalgae cells is very important for free flow of all the organic matter.

Excess carbon source is always routed towards storage lipid pathways so that the energy can be easily available in case of cell dormant phase or unfavorable conditions of cell growth. If a sufficient carbon source is available, the lipid production rate and accumulation will increase up to a maximum level. If the carbon source is limited in the medium, or when the carbon supply gets exhausted from extracellular sources, the competition between the substrates increases leading to demand in such sources which cannot be supplied by traditional autotrophic CO_2 sequestering mechanisms.

In this direction, heterotrophic microalgae have greater advantage in utilization of organic carbon sources. Heterotrophic microalgae are recently receiving a lot of attention in the recent past due to very high amounts of lipids and simultaneous biomass productivity (Chen *et al.*, 1994). The algae have evolved into a stage where they can use simpler as well as complex and diversified organic substrates and efficiently convert them into intracellular lipids and other storage molecules.

The algae are able to break the bond of energy between the substrate molecules and harness the energy released through exergonic processes. This energy can be used tremendously for basic metabolic functions like cell growth, maintenance and locomotion. This function is evident in the present study as the second highest total lipid production (29.5%) was achieved in heterotrophic mode. Many stress conditions ranging from nutrition deficiency (N, P, or Si) to osmotic or temperature changes induce neutral lipid accumulation within cells frequently over 50% of dry cell weight while alleviate cellular growth rates.

SALINITY

Studies have shown that salinity stress can induce higher lipids production along with favorable saturated fatty acids profile (Bahadar and Khan, 2013). Salinity stress is observed when freshwater algae are subjected to higher salt concentration which creates osmotic stress within the cell wall. This usually occurs due to the difference in osmotic potential. Salinity is an intricate stress which influences various physiological and bio-chemical mechanisms associated with the growth and development of microalgae. It can also lead to increment in the total lipid content of microalgae due to its crucial role in causing changes in the fatty acid metabolism. In the present study, the 0.15M salt concentration showed maximum lipid productivity. Under high salinity stress, many organisms including microalgae alter their metabolism to adapt to the extreme environment (VenkataSubash and Venkata Mohan, 2014).

The ability of microalgae to survive in saline environment under the influence of osmotic stress has received considerable attention which can also affect cell growth and lipid formation. When cells are exposed to salinity, specific processes such as, restoration of turgor pressure, regulation of the uptake and export of ions through the cell membrane, and accumulation of osmo-protecting solutes and stress proteins gets activated leading to new and steady state growth (Bahadar and Khan, 2013). These mechanisms in turn, generate stress inside the algal cells causing increment in the total lipid content which act as a reserve energy material until favourable conditions arise (Bahadar and Khan, 2013). Although many species of microalgae are tolerant to great variations of salinity, their chemical and fatty acid composition can vary with respect to salt stress (Wältermann*et al.*, 2005).

NITRATES & PHOSPHATES

Nitrates concentration did not have any influence on total lipid accumulation as it is mainly required for cell division and protein synthesis (Fig. 3.7). Conditions with 400mg/l of nitrates showed total lipid production of 29.5%. Phosphates concentration of 200mg/l showed highest accumulation of total lipids (30.4%) next to 400mg/l at total lipids of (29.5%). Nitrogen and phosphate are two important macronutrients for growth and metabolism of algal cells. Phosphorus is also a key component of phospholipids. It is not unusual for algae to become nutrient-limited (*i.e.*, nitrogen- and phosphorus-limited) in the natural environment. Phosphorus is an important component required for normal growth and development of algal cells (fig 3.18). It has been shown that phosphorus, rather than nitrogen, is the primary limiting nutrient for microalgae in many natural environments. Immediate effects of phosphorus limitation include a reduction in the synthesis and regeneration of substrates in the Calvin-Benson cycle and a consequential reduction in the rate of light utilization required for carbon fixation (Wältermann*et al.*, 2005).

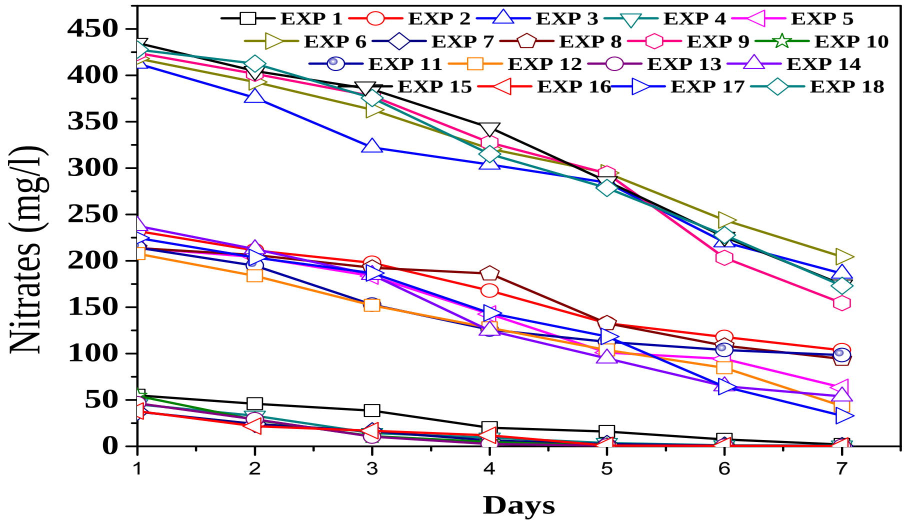

Fig. 3.17 Nitrates removal efficiency.

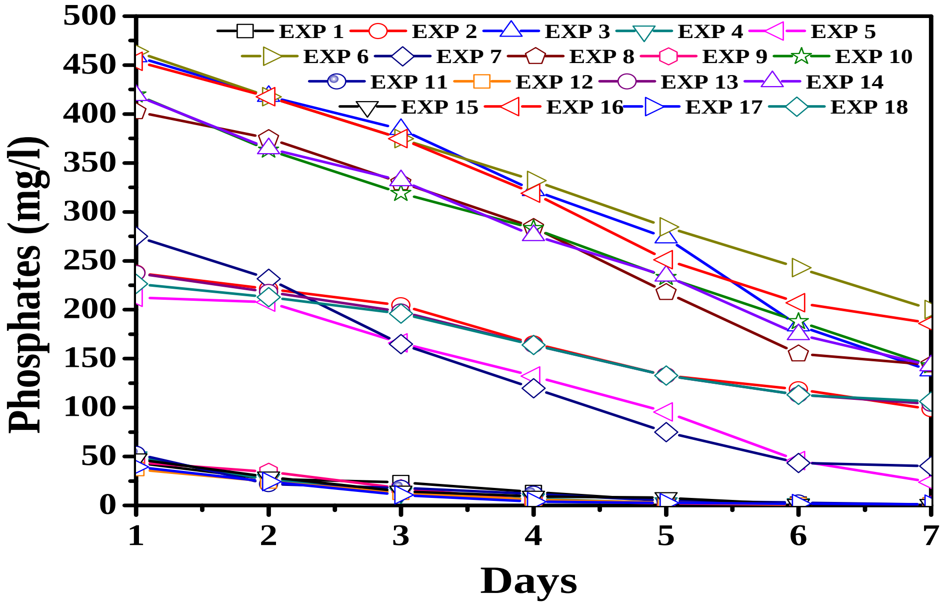

Fig. 3.20 Phosphates removal efficiency

Phosphorus limitation also leads to accumulation of lipids. Total lipid content in *Scenedesmus*sp. was observed to increase from 23% to 53% with a reduction in initial total phosphorus (as phosphate) concentration of 0.1 from 2.0 mg L−1 (Sorokin and Krauss, 1958). Nutrient stress plays critical role in conversion of stored carbohydrates to lipids. Two-phase cultivation was developed to increase lipid accumulation in autotrophic microalgae with starvation (Bahadar and Khan, 2013). It composes of algal growth under sufficient nutrients first for biomass formation then under nutrient deficiency for a several days for the conversion to lipids. Almost two folds increment in lipid productivity was observed during starvation phase operation compared to growth phase (GP).

The carbohydrates stored during SP operation get channelled towards the formation of TAGs. While in GP, the abundant availability of nutrients might have facilitated algae growth rather than lipid accumulation. In the present study, all the conditions where nutrient stress has prevailed showed drastic increments in lipid productivities of total and neutral lipids.

IRON

Trace metals play a critical role in algal metabolism and are required in extremely small quantities (<4 ppm). Iron (Fe) is one of the most important trace metals required by algae for various metabolic functions (Danquah *et al.*, 2009). Deficiencies in iron concentrations can limit algal growth, whereas excesses or high metal concentrations (above the toxicity threshold) may inhibit growth, impair photosynthesis, deplete antioxidants, and damage the cell membrane. It acts as redox catalyst in photosynthesis and nitrogen assimilation and mediates electron transport reactions in photosynthetic organisms (Bahadar and Khan, 2013,Sorokin and Krauss, 1958). Iron limitation significantly supresses photosynthetic electron transfer, resulting in a reduction in NADPH formation. Reduction in iron decreases the cellular abundance of iron containing ferredoxin, a major electron transport molecule. Iron limitation also reduces cellular chlorophyll concentration (Chen *et al.*, 1994). High concentrations of iron in cultures of *Chlorella vulgaris* were observed to increase the lipid content (Venkata Mohan and Devi, 2012).

pH

pH of a microenvironment is important factor which determines the solubility and availability of CO_2 and essential nutrients, and it can have a significant impact on algal metabolism (Venkata Mohan and Devi, 2012). Due to uptake of inorganic carbon by algae, pH can rise significantly in algal cultures (fig 3.19). Maximum total lipids were obtained at pH of 8.5 which was close to optimal algae pH conditions of (8.2). These results are in accordance with Chen and Durbin, where photosynthetic rate and algal growth was minimal at pH 9.0, but carbon uptake rates were enhanced when the pH was lowered to 8.3. This can be attributed to the fact that maximum amount of lipids were obtained in dark conditions where no photosynthetic mechanism prevails.

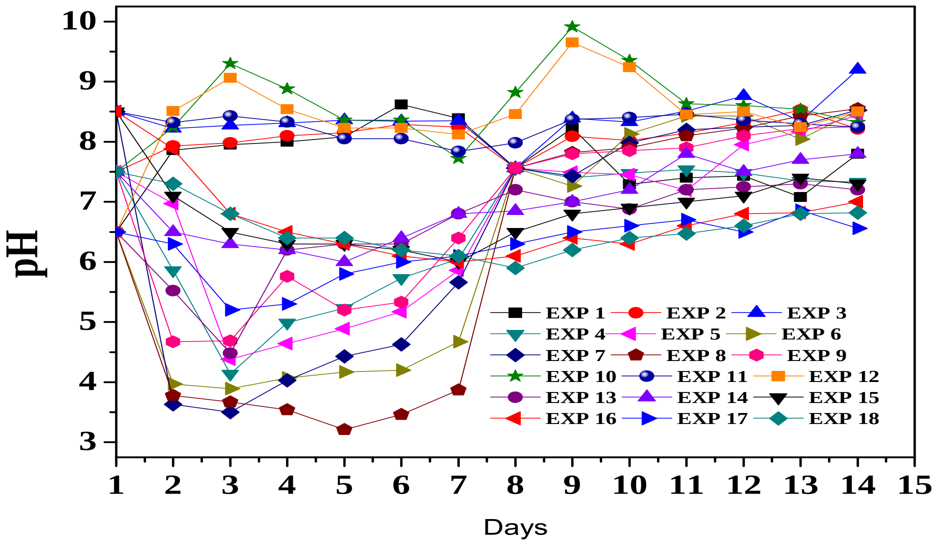

Fig. 3.21 pH influence on lipid production capacities.

3.2 Factor interactions

Optimization of total lipid productivity requires an understanding not only on how various factors individually affect it but also of the multiple interactions that adds to the complexity of the whole process. In this way, the individual parameter interactions, called as severity index (SI), of different factors derived from data analysis helps to understand the influence of the two individual factors at various levels of interaction. Phosphates with trace metals showed highest SI (85.1%) followed by nitrates and light (60.4%) and phosphates with ph (SI 59.3%).

The results showed significant interactions among the factors, with the least SI between nutrient stress and ph (32.6%). Phosphates and trace metals proved to be the most influential factors at interactive levels whereas individually had negligible influence on lipid productivity. Also the presence of phosphates concentration has shown major influence on interactive roles rather than individual components. pH, nutrient stress and salinity have showed negligible influence on interaction level as their role comes into play in the second stage i.e. starvation phase. It is to be noted that all the interactive parameters viz. nitrates, phosphates, trace metals and light have direct influence on biomass growth and subsequent conversion into total lipids present in the cell wall i.e glycolipids and phospholipids. The SI analysis suggested that the nitrates, phosphates, light and trace metals which were of least impact factors at individual levels maximize the total lipid productivity in combination.

Table 3.4: Automated test for presence of interaction factors.

S No	Interacting factor pairs (Based on SI)	Columns	SI (%)	Reserved Column	Optimum levels
1	Phosphates (mg/l) x Trace metals (mg/l)	4 × 5	85.13	1	(3,1)
2	Nitrates ($NaNO_3$) x Light (4000 Lux)	3 × 8	60.45	11	(1,3)
3	Phosphates (mg/l) x pH	4 × 6	59.38	2	(3,3)
4	pH x Salinity (NaCl)	6 × 7	51.45	1	(2,1)
5	Nutrient stress x Trace metals (mg/l)	1 × 5	48.34	4	(1,3)
6	Nutrient stress x Phosphates (mg/l)	1 × 4	44.6	5	(1,1)
7	Carbon supplement x pH	2 × 6	36.1	4	(2,2)
8	Nutrient stress x Salinity (NaCl)	1 × 7	34.32	6	(1,3)
9	Nutrient stress x Carbon supplement	1 × 2	32.84	3	(1,3)
10	Phosphates (mg/l) x Salinity (NaCl)	4 × 7	32.8	3	(3,1)
11	Nutrient stress x pH	1 × 6	32.62	7	(1,3)

3.3 Analysis of variance (ANOVA)

ANOVA with the percentage of contribution of each factor with interactions are shown in Table 3.5. The level of factors to produce the best results can also be observed from Table X. F-ratios suggest that all the factors and interactions considered in the experimental design had statistically significant effects at a 48.4% confidence limit. Experimental degree of freedom (DOF) is 17 while factors–DOF is 2.

Table 3.5: Analysis of variance ANOVA.

S No	Factor	DOF	Sum of squares	Variance	F-Ratio	Pure sum	Percent
1	Nutrient stress	1	122.8	122.8	3,5	88.7	9.3
2	Carbon supplement	2	190.2	95.1	2.7	122.0	12.8
3	Nitrates ($NaNO_3$)	2	111.0	55.5	1.6	42.7	4.5
4	Phosphates (mg/l)	2	46.0	23.0	0.6	0	0
5	Trace metals (mg/l)	2	11.6	5.8	0.1	0	0
6	pH	2	25.0	12.5	0.3	0	0
7	Salinity (NaCl)	2	143.8	71.9	2.1	75.6	7.9
8	Light (4000 Lux)	2	227.3	113.6	3.3	159.0	16.8
Other / Error		2	68.244	34.122			48.41
Total		17	946.358				100.00%

The percentage contribution was calculated for each individual factor by the ratio of pure sum to the total sum of the squares. Among the selected factors, ANOVA indicates that the most influential factor was light, accounting for 16.8% of the overall variance followed by carbon supplementation (12.8%) and nutrient stress (9.3%). pH, phosphates and trace metals showed zero influence on the production process with respect to variance. Altogether, light, carbon and nutrient stress contributed a majority of 38.9% at their individual levels on the total lipid production indicating that these factors played critical role in the optimization.

3.4 Optimum parameters

Optimum conditions to achieve higher lipid productivity from microalgae by the selected factors contribution is shown in Table 3.6. Carbon source was found to be the most significant factor influencing lipid production. Salinity, nitrates, and nutrient stress were the next most important factors followed by phosphates, pH and trace metal concentration. A total contribution of factors at optimized conditions was 21.4% for lipid production with maximum contribution of 4.3% from light. The optimum operating conditions are nutrient stress = yes, 5g/l carbon supplementation, 0 mg/l nitrates concentration, 400 mg/l phosphates, Fe (50mg/l) as trace metal, ph of 8.5, 0 salinity, light intensity of 4000 lux (12:12), were derived from the data analysis resulting in the increment of lipid production from 7.1% to 30.4%.

Table 3.6: Optimum conditions and their contributions.

S.No	Factor	Level description	Level	Contribution
1	Nutrient stress	Yes	1	2.61
2	Carbon supplement	5	2	3.32
3	Nitrates ($NaNO_3$)	0	1	2.60
4	Phosphates (mg/l)	400	3	2.15
5	Trace metals (mg/l)	Fe (50)	3	1.11
6	pH	8.5	3	1.60
7	Salinity (NaCl)	0	1	3.75
8	Light (4000 Lux)	12:12	3	4.30

Total contribution from all factors	21.478
Current grand average of performance	20.876
Expected result at optimum condition	42.354

3.5 Process validation

Experiments performed using the optimum conditions obtained was used to validate the methodology. Results from validation experiments showed an enhanced lipid production of 31% by employing the optimized operating conditions. Significant enhancement in the process performance could be achieved with optimized parameters. Validation results were in good agreement with the parameters that resulted in higher lipid production.

3.6 Bioprocess monitoring

Maximum substrate degradation (based on COD removal efficiency) of 90% was observed along with lipid synthesis. Variation in the lipid synthesis in the cytoplasm by microalgae is mainly dependent on the type of nutritional mode (autotrophic, mixotrophic and heterotrophic) which depends on the availability of carbon and nutrients. Microalgae can also function under mixotrophic mode by combining both the autotrophic and heterotrophic mechanisms (Bahadar and Khan, 2013). Mixotrophic growth can be defined as the simultaneous assimilation of both CO_2 and organic carbon leading to the concurrent operation of respiratory and photosynthetic metabolisms. Mixotrophic cultures show reduced photo inhibition and improved growth rates over autotrophic and heterotrophic cultures (Bahadar and Khan, 2013).Organotrophic means the synthesis of reserve food materials from pre-existing organic sources (starch, glucose and carbohydrates). Two carbon acetyl-CoA molecules are the major components which initiate the lipid metabolism produced either by photosynthetic mechanism in light assisted conditions (12:12h) or hexose/H+ symport system followed by oxidative pentose phosphate pathway (OPPP).

4. CONCLUSIONS

The Taguchi design of experimental methodology illustrated its application for process optimization based on selected critical factors of microalgae lipid production. The methodology also facilitated to understand the specific functional role of eight factors that influence lipid production by microalgae. Among the eight factors, carbon supplementation individually showed significant influence on the lipid synthesis, followed by salinity, nitrates, nutrient stress, light, phosphates, pH and trace metals. The validation experiments performed with the obtained optimized conditions showed enhanced total lipid production of 31.9%.

The potential of photosynthetic microorganisms (microalgae) towards bioenergy generation in the form of biodiesel showed promising results as they have the ability to sequester carbon, higher lipid accumulation, easy cultivation and wastewater treatment. From these results, we conclude that micro algal oil is a source of renewable energy production.

REFERENCES

1. Abdullah, A.Z.; Salamatinia, B.; Mootabadi, H.; Bhatia, S, Energy Policy, 2009; 37: 5440-5448.
2. Abou-Shanab, R.A.I.; Hwang, J.-H.; Cho, Y.; Min, B.; Jeon, B.H, Applied energy, 2011; 88: 3300-3306.
3. Abou-Shanab.; AIReda.; Matter AIbrahim.; Kim SN.; Oh You-Kwan.; Choi Jaeyoung.; Jeon Byong-Hun, Biomass Bioenergy, 2011; 1-7.
4. Bahadar A.; Khan MB, A review Renewable and Sustainable Energy Reviews, 2013; 128-148.
5. Bajguz A, Journal of Plant Physiology, 2009; 882-886.
6. Banerjee, A.; Chakraborty, R, Conservation and Recycling, 2009; 53: 490-497.
7. Berges JA.; Varela DE.; HarrisonPJ, 2002; 139–146.
8. Buckle KA.; Edwards A, puree, 1970; 173–186.
9. Canakci, M, Bioresource Technology, 2007; 98: 183-190.
10. Chen CY.; Durbin EG, Mar. Ecol.-Prog. Ser, 1994; 109: 83–94.
11. Chinnasamy S.; Ramakrishnan B.; Bhatnagar A.; Das KC, International Journal of Molecular Sciences, 2009; 518-532.
12. Danquah MK.; Gladman B.; Moheimani N.; Forde GM, Chemical Engineering Journal, 2009; 151: 73-78.
13. Demirbas, A, Springer – Verlag, London, 2008.
14. Devi MP.; VenkataSubhash G.; Venkata Mohan S, Ren Energy, 2012; 276–283.
15. Farrell AE.; Plevin RJ.; Turner BT.; Jones AD.; O'Hare M.; Kammen DM, Science, 2006; 311: 506-8.
16. Goldman JC, Science, 1973; 182: 306–307.
17. Guckert JB.; Cooksey KE, J. Phycol, 1990; 72–79.
18. Harwood JL, Function and Genetics; Siegenthaler, P.A., Murata, N., Eds, 2004; 287–302.
19. Islam R.; Hassan A.; Sulebele G.; Orosco C.; Roustaian P, Int. J. Sci, 2003; 97-106.
20. Ivashin, N.; Källebring, B.; Larsson, S.; Hansson, Ö.; J. Phys. Chem, 1998; 102: 5017–5022.
21. Jain, S.; Sharma, M.P, Renewable and Sustainable Energy Reviews, 2010; 14: 763-771.
22. Jeffrey SW.; Humphrey GF, Biochem. Physiol. Pflanz, 1975; 191–194.
23. Juneja A.; Ceballos RM.; Ganti S, A Review, 2013; 4607-4638.
24. Kang, S.W.; Park, Y.S.; Lee, J.S.; Hong, S.I.; Kim, S.W, Bioresour. Technol, 2004; 91: 153–156.
25. Lee, R.E, Phycology. Forth ed. Cambridge University Press, Cambridge, 2008.
26. Leeuwen JV.; Rasmussen ML.; Sankaran S, Koza CR.; Erickson DT.; Mitra D.; Jin B, Sustainable Bioenergy and Bioproducts, 2012; 13-44.
27. Li Y.; Zhao Z.; Bai F, Enz Microbiol Technol, 2007; 1: 312–7.
28. Ma FR.; Hanna MA, Bioresour Technol, 1999; 70: 1-15.
29. Mangos TJ.; Berger RG, Europian food research and technology, 1997; 45-350.
30. Mata, T.M.; Martins, A.A.; Caetano, N.S, Renewable and Sustainable Energy Reviews, 2010; 14: 217-232.
31. Mata, T.M.; Martins, A.A.; Caetano, N.S, Renewable and Sustainable Energy Reviews, 2010; 14: 217-232.
32. Meher, L.C.; Naik, S.N.; Naik, M.K.; Dalai, A.K, CRC Press. Boca Raton, 2008; 255-266.
33. Pisciotta, J.M.; Zou, Y.; Baskakov, I.V.; Plos one, 2010; 5: 1–10.
34. Pruvost, J.; Van Vooren, G.; Cogne, G.; Legrand, J, Bioresource Technology, 2009, 100, 5988-5995.
35. Raven JA.; Geider RJ, New Phytol, 1988; 110: 441–461.
36. Sheehan, J.; Dunahay, T.; Benemann, J.; Roessler, P, National Renewable Energy Laboratory, 1998.
37. Solovchenko AE, Russian Journal of Plant Physiology, 2012; 167–176.
38. Sorokin, C.; Krauss, R.W, Plant Physiol, 1958; 33: 109–113.
39. The state of food and agriculture. BIOFUELS: prospects, risks and opportunities, 2008.
40. Venkata Mohan S.; Chandrasekhara Rao N,; Krishna Prasad K.; Muralikrishna P.; Sreenivasa Rao R.; Sarma PN, Biotechnol Bioeng, 2005; 90: 732–45.
41. Venkata Mohan S.; Devi MP, 2014, http://dx.doi.org/10.1016/j.biortech.2014.02.103.
42. Venkata Mohan S.; Devi MP, Bioresource Technology, 2012; 627-635.
43. Venkata Mohan S.; Devi MP, Bioresource Technology, 2012; 627-635.
44. Venkata Mohan, S.; Prathima Devi, M.; Mohanakrishna, G.; Amarnath, N.;, Lenin Babu, M.;

Sarma, P.N, Bioresour. Technol, 2011; 102: 1109–1117.

45. Venkata Mohan, S.; Prathima Devi, M.; VenkataSubhash, G.; Chandra, R. 2014. Algae oil as fuels. Bioresour. Technol.
46. Venkata Mohan, S.; Veer Raghavulu, S.; Sarma, P.N, Biosens. Bioelectron, 2008; 24: 41–47.
47. VenkataSubhash G.; Venkata Mohan S, Fuel, 2014; 509–515.
48. Vicente G.; Martinez M.; Aracil J, BioresourTechnol, 2004; 92: 297-305.
49. Wältermann M.; Hinz A.; Robenek H.; Troyer D.; Reichelt R.; Malkus U.; Galla H J.; Kalscheuer R.; Stöveken T.; Landenberg P.; Steinbüchel A, Molecular Microbiology, 2005; 750–763.