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# Salinity stress induced lipid synthesis to harness biodiesel during dual mode cultivation of mixotrophic microalgae

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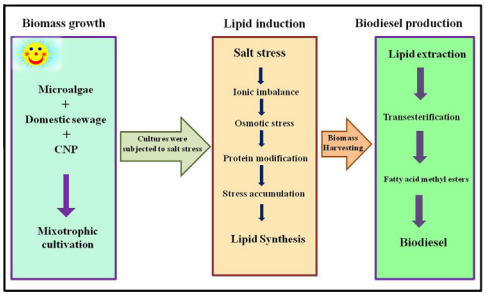
## HIGHLIGHTS

- Dual mode cultivation strategy for maximization of biomass and lipid productivity.
- Salinity stress showed positive impact on lipid synthesis.
- Diverse fatty acid profile was detected with the function of salinity stress.
- Carbohydrate profile showed direct correlation with the lipid productivity pattern.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Influence of salinity as a stress factor to harness biodiesel was assessed during dual mode cultivation of microalgae by integrating biomass growth phase (BGP) and salinity induced lipid induction phase (LIP). BGP was evaluated in mixotrophic mode employing nutrients (NPK) and carbon (glucose) source while LIP was operated under stress environment with varying salt concentrations (0, 0.5, 1 and 2 g NaCl/l). Salinity stress triggered both biomass growth and lipid synthesis in microalgae significantly. BGP showed higher increments in biomass growth (2.55 g/l) while LIP showed higher lipid productivity (1 g NaCl/l; total/neutral lipid, 23.4/9.2%) than BGP (total/neutral lipid, 15.2/6%). Lower concentrations of salinity showed positive influence on the process while higher concentrations showed marked inhibition. Salinity stress also facilitated in maintaining saturated fatty acid methyl esters in higher amounts which associates with the improved fuel properties. Efficient wastewater treatment was observed during BGP operation indicating the assimilation of carbon/nutrients by microalgae.



## 1. Introduction

Microalgae are considered to be the efficient photosynthetic organisms that have been suggested as a potential feedstock for the production of biofuels and bioenergy (Klein et al., 2013; Alcantara et al., 2013). Microalgae produce biofuels and other chemical by harvesting sunlight and fixing $CO_2$. The fixed $CO_2$ within these cells under stress conditions leads to the formation of lipids that can be transesterified to produce biodiesel (Rawat et al., 2013; Venkata Mohan et al., 2013). Lipids are the secondary metabolites of microalgae synthesized during stress conditions (Solovchenko, 2012; Devi et al., 2013; Venkata Mohan et al., 2013). Lipids, in the form of triacylglycerides typically provide a storage function in the cell that enables microalgae to endure adverse environmental conditions (Giorgos and Elias, 2013; Kalpesh et al., 2012). Studies have indicated that the lipid content of microalgae can be enhanced by changing the cultivation conditions and

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subjecting them to diverse stress conditions (Pittman et al., 2011; Devi and Venkata Mohan, 2012; Asulabh et al., 2012; Chandra et al., 2014). Among the cultivation methods, two-stage systems are of intense interest where, optimum conditions are applied in the first phase aiming at the maximization of biomass production, while in the second phase, stress conditions are applied to facilitate the accumulation of lipids (Giorgos and Elias, 2013; Devi et al., 2012; Devi and Venkata Mohan, 2012. The major stress conditions applied to enhance lipid accumulation are temperature, light intensity, pH, salinity, mineral salts and nutrients (Takagi, 2006; Ifeanyi et al., 2011; Devi et al., 2012).

Salinity is an intricate stress which influences various physiological and bio-chemical mechanisms associated with the growth and development of microalgae. Salinity stress can also lead to increment in the lipid content of microalgae due to its crucial role in causing changes in the fatty acid metabolism (Kalita et al., 2011). Under high salinity stress, many organisms including microalgae alter their metabolism to adapt to the extreme environment (Kan et al., 2012). 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 (Asulabh et.al., 2012). Fluctuations in the salt content of the growth medium has also been found to alter the lipid composition of microalgae (Kalpesh et al., 2012). As, algae are inhabitants of biotopes characterized by varying salinities, they have gained significance in salt tolerance studies domain and have served as model organisms for better understanding of salt acclimation in more complex physiological processes (Talebi et al., 2013; Alkayal et al., 2011). 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 steady state growth (Talebi et al., 2013; Allakhverdiev et al., 2000). These mechanisms in turn, generates stress inside the algal cells causing increment in the total lipid content which act as a reserve energy material until favourable conditions arise (Talebi et al., 2013; Asulabh et.al., 2012). *Dunaliella* sp. provide the best example of microalgae that can tolerate high salt concentrations (Azachi et al., 2002). Their ability to increase the biomass growth and lipid content under salinity stress makes them one of the suitable candidates to study the effects of salinity on microalgae (Kalpesh et al., 2012). Although many species of microalgae including marine heterotrophic strains are tolerant to great variations of salinity, their chemical and fatty acid composition can vary with respect to salt stress (Kalpesh et al., 2012; Kirroliaa et al., 2011). The effect of salt concentration was evaluated to explore the potential of marine resources confined with specific species of microalgae (Ifeanyi et al., 2011; Takagi, 2006). When photosynthetic organisms are exposed to salt stress, the fatty acids of membrane lipids are desaturated leading to increment in the proportion of unsaturated fatty acids (Asulabh et al., 2012). On the other hand, increment in the saturated fatty acids and decrement in polyunsaturated fatty acids under high-salt stress has also been reported (Kan et al., 2012). Algae produce some metabolites to protect them from salt injury and also to adjust to the surrounding osmotica (Rao et al., 2007).

In the present study, the role of salinity as a stress factor on microalgae lipid synthesis towards biodiesel production was evaluated in dual mode cultivation viz., biomass growth phase (BGP) followed by lipid induction phase (LIP). BGP was evaluated in mixotrophic mode employing domestic wastewater as substrate to induce growth while LIP was operated in autotrophic mode by varying the concentrations of sodium chloride. Changes in the biomass growth, chlorophyll components, lipid productivities and fatty acid compositions were studied at the end of both BGP and LIP operations. Wastewater treatment capability of the system was also evaluated at the end of BGP operation.

## 2. Methods

### 2.1. Microalgae

Microalgae culture collected in pre-monsoon season from a lentic water body (Nacharam Cheruvu, Hyderabad) receiving domestic effluents was used as inoculum (Venkata Mohan et al., 2011). Prior to experimentation, the culture was washed and pelletized by centrifugation (3000 rpm; 10 min; 30 °C) to remove associated debris.

### 2.2. Experimental methodology

Experiments were designed and operated in dual mode viz., biomass growth phase (BGP, mixotrophic) followed by salinity stress induced lipid induction phase (LIP, autotrophic). In BGP, microalgae were grown mixotrophically in culture-tub (working volume, 40 L; depth, 10 cm; surface area, 0.23 $m^2$) and maintained as an open-pond system. The culture-tub was fed with domestic sewage (COD, 400 mg/l; TDS, 750 mg/l; nitrates, 115 mg/l) with additional supplementation of carbon (500 mg glucose/l) and nutrients (500 mg $NaNO_3$/l; 500 mg $Na_2PO_4$/l) to accelerate algal biomass growth by adjusting pH to 8.2. After 8 days of growth period, the cultures were harvested by siphoning wastewater from the tub. The dewatered culture was used as inoculum for the second phase (LIP) of the experiments at where, three levels of sodium chloride viz., 0.5, 1.0 and 2.0 g NaCl/l along with a control condition without NaCl were operated to assess the influence of natural stress (NS). Prior to start-up, 20 ml of microalgal biomass (2.14 g/l) was inoculated to 160 ml of tap water and closed with cotton plugs. Before inoculation, the pH was adjusted to 8.2 using 3 N NaOH. LIP-NS condition was operated with tap water alone. Experiments were carried out under 12 h (light):12 h (dark). In the light phase, flasks were mounted on a temperature controlled shaking incubator (120 rpm) in the presence of a fluorescent light (0.074 mol/$m^2$ s). In dark phase, the light source was turned off to facilitate dark conditions while the rest of the operating conditions remained same. After LIP, the resulting biomass was separated by centrifugation (5000 rpm; 5 min at 28 °C) and the algal biomass pellet was subjected to solar drying followed by blending in to powder form. The blended powder was further disrupted using sonicator (20 kHz) for 30 min (Power Sonic 410) and the extracted lipid was used for analysis. All the experiments were carried out in triplicates and the results presented here represent an average of three independent operations.

### 2.3. Extraction of lipids

Extraction of total lipids was carried out using Bligh and Dyer method employing chloroform:methanol (2:1 v/v) as solvents while neutral lipids were extracted with n-Hexane employing solvent extraction procedure using Soxhlet apparatus (Lee et al., 2010; Venkata Mohan et al., 2011). Prior to extraction, the dried algae-biomass was sonicated (30 min; 20 kHz) in requisite solvent / solvent mixture (chloroform:methanol/n-Hexane) and transferred to thimbles made with Whattman filter paper No. 1 for neutral lipids where as the biomass was directly placed in to the reaction vials for total lipids extraction. The solvent mixture was refluxed for 5 h (30 °C) and concentrated in a rotavapor followed by vacuum drying with a temperature controlled oil bath (120 °C) and cooled to room temperature (Devi and Venkata Mohan, 2012).

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