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Modulation of biodiesel production by sodium bicarbonate and nitrogen deficiency in the microalga *Chlorella vulgaris*

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ABSTRACT

Algal biodiesel is a sustainable and a renewable biofuel. Therefore, it is important to optimize the lipid production in microalgae. To enhance bio-diesel production the unicellular alga *Chlorella vulgaris* was grown in ambient air and high CO_2 (5% CO_2, rest air) in the absence or presence of $NaHCO_3$ (12 mM) that provides additional carbon source for algal photosynthesis and biomass production. In ambient air the algal biomass increased 2.5 fold in the presence of $NaHCO_3$. High CO_2 purging of the algal growth medium in the presence of $NaHCO_3$ partially increased the biomass by 10%. In the same vein the chlorophyll content of algal culture increased (100% to 139%) due to the abundant availability of the carbon source. Addition of $NaHCO_3$ increased biodiesel amount by 12%. These demonstrate that addition of extra carbon source i.e., $NaHCO_3$ that is readily photosynthetically fixed by the coordinated action of carbonic anhydrase and rubisco increases the carbon skeleton to be used to optimize biodiesel production by microalgae. Further, the nitrogen deficiency decreased the algal biomass due to reduced N assimilation and amino acid synthesis. The carbon skeletons in N-deficient culture were diverted towards fatty acid synthesis that resulted in 50% increase in lipid content i.e., from 40% to 60% per algal dry cell weight. This approach could be further optimised for large scale biodiesel production.

KEY WORDS: BIODIESEL, *CHLORELLA VULGARIS*, $NAHCO_3$ SUPPLEMENTATION, NITROGEN DEFICIENCY

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INTRODUCTION

Microalgae are fast-growing photosynthetic organisms that fix CO_2 to generate millions of tons of algal biomass in nature. In addition to photosynthetic production of carbohydrates, several microalgae synthesize lipids by using carbon skeletons and reducing equivalents generated from carbohydrate metabolism. Lipids extracted from green algae can be utilized for biodiesel generation, and whatever is left of the biomass can be changed over into ethanol that may be used as biofuel. Biodiesel generation from microalgae is a desirable option. However, their lipid content is required to be high to be an economically viable option. The green alga *Chlorella vulgaris* is widely studied for biodiesel production (Chiu et al., 2008). It has higher productivities than land plants and generates a lot of fatty acids the precursors of biodiesel. To grow algae crop land is not required. It can be grown in ponds or lakes. However, a few challenges need to be overcome to use algae as a sustainable and economically viable option for commercial biodiesel production from *Chlorella*. Microalgae use bicarbonate as the exogenous carbon source for photosynthesis. $NaHCO_3$ is converted to CO_2 via carbonic anhydrase (Dixon et al., 1987; Nimer et al., 1997). Algae could use purged high CO_2 in the growth medium for photosynthesis via same mechanism (Baldisserotto et al., 2014). Nitrogen is a main element controlling species component, diversity, and primary productivity of species (Mandal et al. 2018). Limiting or starving the availability of essential nutrients such as nitrogen (N) can induce triacylglycerol's (TAGs) accumulation (Janssen et al. 2019).

In the present study, we have optimized biomass generation and lipid production of *Chlorella* by providing $NaHCO_3$ as an inorganic carbon source. In addition we have purged the growth medium with ambient air or high CO_2 (5% CO_2, rest air) in the absence or presence of $NaHCO_3$ as additional carbon source for photosynthetic carbon assimilation. These cells were further grown in the N-deficient conditions to increase their lipid content. We have shown than addition of $NaHCO_3$ doubled the algal growth and increase biomass production when purged with either air or 5% CO_2. Their lipid content increase by 12% by $NaHCO_3$ and it further increased in N-deficient growth condition by 50%.

MATERIAL AND METHODS

Photobioreactor and cultivation conditions: *Chlorella vulgaris* (an isolate of river Ganges, courtesy: Prof. Nirupama Mallick, Indian Institute of Technology, Kharagpur, India), was used in this research. Pure culture of *C. vulgaris* were grown in N11 medium (Soeder and Bolze, 1981) at pH 6.8 and maintained in a culture room at 25 ± 2˚C under a photoperiod of 14:10 h at a light intensity of 150 µmol photon m^{-2} s^{-1}. The photobioreactor was setup in a temperature-controlled environment fitted with nine LED fluorescent lamps (Philips, 36W) containing 6 photobioreactor units. Photobioreactors were 1.5 L borosilicate glass tubes with a working- volume of 1.3 L, fitted with sealed stoppers and autoclaved as complete units. Airflow into the photobioreactor was supplied via filtered hydrocarbon-free building air and 5% CO_2 through teflon tubing at a rate of 200 mL min^{-1} (i.e., 0.25 vvm, volume gas per volume media per min). The airflow was adjusted using a stainless steel micrometering valve (JTM LPA Air, Japsin Rotameter). Experimental replication was achieved by repeating each treatment 4 times. The initial biomass concentration for all cultures was 0.11 g L^{-1} and all runs studied for 14 days. When required 12 mM $NaHCO_3$ was added to the growth medium to increase the carbon source for biomass and biodiesel production. After addition of $NaHCO_3$ the pH was adjusted to 6.8. To cause 90% N deficiency (N10), 90% of KNO_3 was substituted by KCl.

Studies on nitrogen deprivation with and without $NaHCO_3$: To study the effects of nitrate, starvation, algal cultures pre-grown in N 11 medium were harvested by centrifugation, washed with N 11 medium without the specific nutrient for 2-3 times and transferred to the 90% N-deficient medium (N10). *C. vulgaris* was grown in ambient or 5% CO_2 without or with $NaHCO_3$ (12 mM) as additional carbon source to support photosynthesis. Cultures were grown under bicarbonate supplementation into early stationary growth phase (10–12 days) and samples were taken for analysis at desired time point to monitor biomass production, chlorophyll content and cellular lipid content.

Analytic determinations: Dry cell weight (dcw) was measured gravimetrically (Rai et al., 1991). To determine the quantity of total chlorophyll, extraction was done according to Sartory and Grobbelaar (1984) with small modifications. To 1 mL of pellet and beaded culture, 2 mL of methanol was added and heated for 10 min at 70 °C in a water bath in a sealed tube. After cooling, the samples were centrifuged for 10 min (4 °C; 6,000g). For chlorophyll a, chlorophyll b quantification, absorbance was recorded for the supernatants at 666 nm, 653 nm and estimation was done using the equations proposed by Wellburn (1994). Extraction of lipid from the dried biomass was done following the protocol of binary solvents using chloroform and methanol (Bligh and Dyer, 1959).

RESULTS AND DISCUSSION

In the present study, the $NaHCO_3$ was given as an inorganic carbon source for algal growth. Simultaneously, it

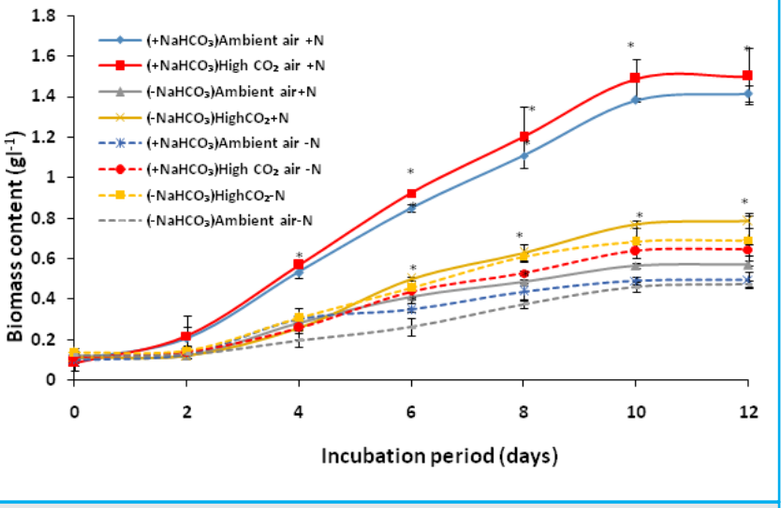

FIGURE 1. Modulation of *Chlorella vulgaris* biomass by $NaHCO_3$, high CO_2 and nitrogen deficiency. Algae were grown in N11 media with or without $NaHCO_3$ and high CO_2 in the N-deficient (N10) or N-sufficient conditions. Two ml of algal culture were taken from the bioreactor on alternate days and their dry biomass was determined as described in Methods. Each data point is the average of 3 replicates and the error bars represent sd. Asterisks indicate significant differences determined by t-test (*P<0.05)

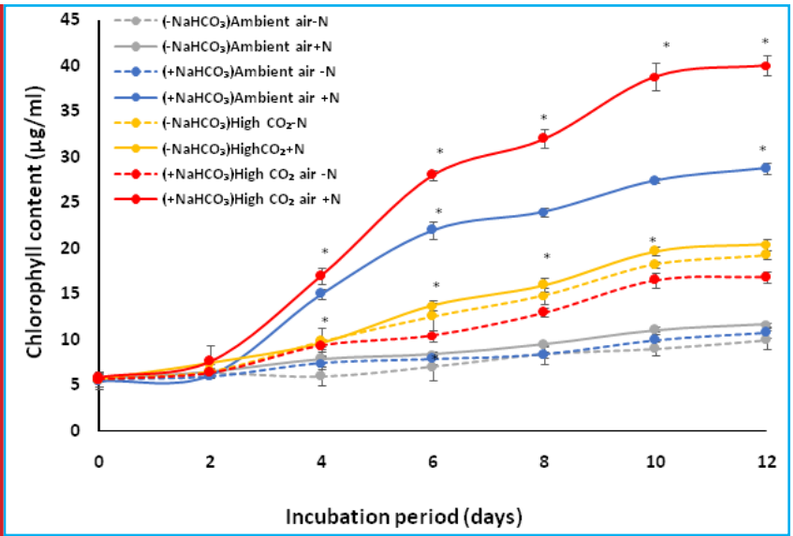

FIGURE 2. Chlorophyll content of *C. vulgaris* culture supplemented with and without bicarbonate in N-deficient and N-sufficient growth media. Algae were grown in N11 media with or without $NaHCO_3$ and high CO_2 in the N-deficient (N10) or N-sufficient conditions. One ml of algal culture was taken from the bioreactor on alternate days and their chlorophyll content was measured by spectrophotometry as described in Methods. Each data point is the average of 3 replicates and the error bars represent sd. Asterisks indicate significant differences determined by t-test (*P<0.05) In N-deficient (N10) growth condition the pigmentation of algal cells declined by 18%, 5.5%, 62% and 57% in -$NaHCO_3$ ambient air, -$NaHCO_3$ high CO_2, $NaHCO_3$ ambient air and +NaHCO3 5% CO_2 growth media respectively. Loss of Chl was previously reported in algal cells due to nutrient deficiency (Fredeen et al. 1990; Jacob and Lawlor 1993; Lippemeier et al. 2003).

was bubbled with air or 5% CO_2 to increase the carbon source. As shown in figure- 1, the algal growth, determined as dry biomass accumulation, saturated after 10 days of growth. The algal cells grown in the photo-bioreactor purged with ambient air accumulated 0.57 g of dry biomass l^{-1} on10th day of growth Upon purging the culture medium with 5% CO_2, the growth and biomass accumulation increased by 38%.

The biomass accumulation substantially increased in the presence of additional inorganic carbon source $NaHCO_3$. When the growth medium was supplemented with 12 mM $NaHCO_3$ and bubbled with air the biomass accumulation substantially increased i.e., by 2.5 fold to 1.41 g l^{-1}. Upon purging the $NaHCO_3$ supplemented growth medium with 5% CO_2 the biomass accumulation further increased to 1.5 g l^{-1}. This accumulation was 100% higher than biomass produced in high CO_2 grown minus HCO_3 samples. This demonstrates that by providing additional inorganic carbon source the algal growth could almost be doubled. Bubbling high CO_2 alone does not achieve the desired result. Our results further demonstrate that high CO_2 purging of algal culture could partially (8%) increase algal growth in the presence of 12 mM $NaHCO_3$. Increase of carbon source as $NaHCO_3$ could provide high CO_2 needed by Rubisco to efficiently fix carbon by the photosynthetic Calvin-Benson cycle. $NaHCO_3$ is not the substrate of Rubisco, rather it is CO_2. The $NaHCO_3$ would have converted to CO_2 by intracellular carbonic anhydrase (Raven, 1995) before its fixation by Rubisco.

Nitrogen deficiency is known to cause reduction of algal growth (Wen *et al.* 2003; Hejazi *et al.* 2004; Aro 2016). To probe the role of nitrogen deficiency on algal growth supplemented with additional sources of inorganic carbon, we monitored the biomass of *C vulgaris* in the absence or presence of $NaHCO_3$ + 5% CO_2 in 90% N-deficient media (Fig. 1). Due to N deficiency, i.e., in 90% N deficient state (N10), the biomass of algal culture declined by 17%, 11%, 62% and 57% in -$NaHCO_3$ ambient air, -$NaHCO_3$ high CO_2, +$NaHCO_3$ ambient air and +$NaHCO_3$ 5% CO_2 growth condition respectively. The highest percentage (61%) of reduction of biomass production in N -deficient condition was observed in high CO_2-grown and $NaHCO_3$-supplemented algal cells. These clearly suggest that N is a highly limiting factor for algal growth in the presence of high inorganic carbon sources. The nitrogen deficiency decreased the algal biomass due to reduced N assimilation and amino acid and protein synthesis. The reduction in biomass was predominantly due to reduced cell division in N-limited growth. This is in agreement with previous studies with *Chlamydomonas reinhardtii and Scenedesmus subspi-*

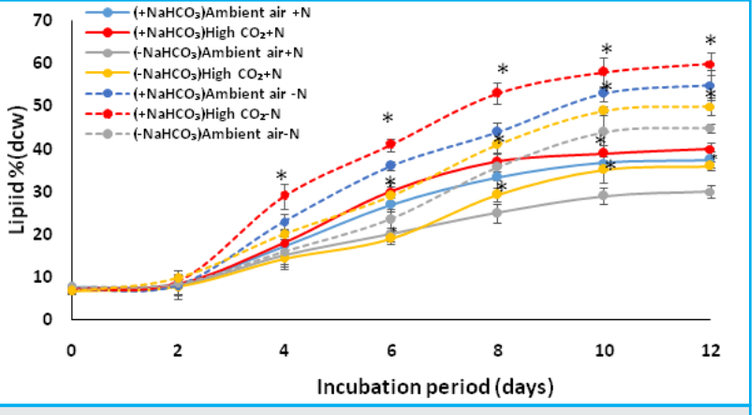

FIGURE 3. Impact of $NaHCO_3$ and high CO_2 and nitrogen deficiency on cellular lipid % (dry cell weight) in Chlorella vulgaris. Algae were grown in N11 media with or without $NaHCO_3$ and high CO_2 in the N-deficient (N10) or N-sufficient conditions. Fifty ml of algal culture were taken from the bioreactor on alternate days and their dry biomass was determined as described in Methods. Each data point is the average of 3 replicates and the error bars represent sd. Asterisks indicate significant differences determined by t-test (*P<0.05). The carbon skeletons in N-deficient culture were used towards fatty acid synthesis that resulted in 50% increase in lipid content i.e., from 40% to 60% per algal dry cell weight. The N deficiency possibly upregulated the genes responsible for fatty acid biosynthesis. In the absence of aminoacid synthesis this would have targeted carbon precursors generated in photosynthesis to be redirected towards fatty acid synthesis.

catus where nitrogen deficiency significantly inhibited their cell division (Dean et al., 2010).

As shown in figure 2, the chlorophyll content of algal cells after 10 days of grown in high CO_2 was higher than ambient. In the presence of $NaHCO_3$ the chlorophyll content of algal cells substantially increased (100% - 139%). This was probably owing to increased number of cells due to higher cell division in high CO_2 environment and higher Chl content per cell.

Addition of $NaHCO_3$ increased the lipid amount by 12%. These demonstrate that addition of extra carbon source i.e., $NaHCO_3$ that is fixed photosynthetically by the coordinated action of carbonic anhydrase and Rubisco to generate carbohydrates and carbon backbones could be used for lipid production by microalgae. Limitations of nitrate, under ambient and high (5%) CO_2 stimulated lipid accumulation (Fig. 3) in the microalga *C. vulgaris*. Lipid percent of dry cell weight increased up to 10^{th} day of culture both in ambient and high CO_2. N deficiency is known to increase lipid content i.e., triacylglycerol in algal cells (Illman et al., 2000) (Janssen et al. 2019). On 10th day of culture, the lipid percent of dry cell weight (dcw) of algal culture in N-deficient (N10) growth condition increased by 51.7 %, 40%, 47% and 48% in -$NaHCO_3$ ambient air, -$NaHCO_3$ high CO_2, $NaHCO_3$ ambient air and +$NaHCO_3$ 5% CO_2 growth media respectively.

CONCLUSION

Chlorella vulgaris grown photo-autotrophically is an excellent organism to generate biodiesel. The growth and biomass of the algal culture nearly doubled by adding $NaHCO_3$ (12 mM) in high CO_2 (5%) growth condition. The lipid production increased by 50% due to N deficiency. The growth of *Chlorella vulgaris* in high CO_2, $NaHCO_3$ in N-deficient conditions could be further optimized for large scale production of biodiesel in an industrial scale.

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Conflict of interest

Authors have declared no conflict of interest.

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