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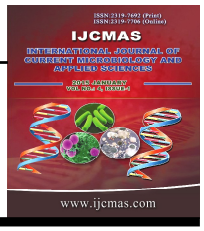

Original Research Article

Effect of Various Carbon Sources on Biochemical Production in Marine Microalgae *Nannochloropsis salina* (Eustigmatophyceae), *Dunaliella tertiolecta* (Chlorophyceae) and *Tetraselmis suecica* (Chlorodendrophyceae)

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A B S T R A C T

The present study involves three different marine microalgal Biochemical productions (*Nannochloropsis salina, Dunaliella tertiolecta* and *Tetraselmis suecica)* in response to the various carbon sources (glucose, fructose, sucrose, lactose and galactose) of 10gL^{-1} in F2 media, respectively. The cultures with controls were maintained under 25 ± 1°C at 150 µmol photons/m^2/s of light intensity and 12:12 light/dark photo period of 30 days. Among the carbon sources tested, the maximum growth rate (K), lipid accumulation, protein and carbohydrate (µg/ml) were found of 0.5065, 143.64 ± 6.15, 61.52 ± 3.06 and 125.83 ± 5.02 (*N. salina)*, 0.3122, 123.57 ± 5.55, 89.48 ± 3.25 and 93.77 ± 4.34 (*D. tertiolecta)*, 0.2319, 123.83 ± 5.55, 117.07 ± 3.65 and 113.65 ± 4.73 (*T. suecica)* respectively, using glucose as carbon source. These values were respectively higher than those obtained in the corresponding photoautotrophic control cultures. The biomass yield and lipids contents were considerably increased with sucrose and fructose in relation to controls.

Keywords

Biodiesel, Biomass, Fatty acid, Sugars, Protein

Introduction

Recently, fuel production from algae has been receiving considerable attention because of growing energy prices, emissions of green house gases (Xiong *et al.,* 2010) and gradual depletion of fossil fuels (Damiani, 2010). Microalgae due to their rapid biomass production, high photosynthetic efficiency (Xiong *et al.*, 2010) and ability to storage a large amount of lipid are ideal source of biodiesel. In fact, microalgae have the highest lipid yield among various oil plants, and the lipid content of some microalgae has up to 80% and the compositions of microalgal oils are mainly TAG which is the right kind of oil for producing biodiesel (Chisti, 2007; Amin, 2009). Now days, production of microalgal biomass can be achieved by photoautotrophic cultivation, using sun light and CO_2, and heterotrophic cultivation using

organic carbon source, it has several advantages over photoautotrophic cultivation including elimination of light, good control of cultivation process, high biomass and lipid content in cells (Miao and Wu, 2006; Huang *et al.,* 2010). However, to expand this novel feedstock, research and development is needed in several domains, from the screening of suitable strains to the standardization of production process as well as the low cost of cultivation process to obtain a large amount of biomass and lipid productivity. Jin Liu *et al.* (2010) reported *C. zofingiensis* could grow rapidly in the dark with glucose as the sole carbon and energy source. Furthermore, by using fed-batch culture strategy, up to 53 g L^{-1} biomass could be reached for this alga (Sun *et al.,* 2008).

In this study the biomass yield, lipid, protein and carbohydrates production from marine microalgae *Nannochloropsis salina*, *Dunaliella tertiolecta* and *Tetraselmis suecica* by supplementing a standard inorganic medium with various organic carbon sources (glucose, fructose, sucrose, lactose and galactose) were examined respectively, and the possibility of growth in heterotrophic culture by this microalgae with these nutrients.

Materials and Methods

Microalgae strain

Nannochloropsis salina, Dunaliella tertiolecta and *Tetraselmis suecica* were obtained from the Central Institute of Brackishwater Aquaculture (OIC, Muttukadu, Chennai, India). These algae were maintained at 4°C on agar slant of f/2 medium (Guillard and Ryther, 1962) consisting of (per litre) 0.75 g $NaNO_3$; 0.005g $NaH_2PO_4.H_2O$; 0.03g $Na_2SiO_3.9H_2O$; 3.15g $FeCl_3.6H_2O$; 4.36g $Na_2EDTA.\ 2H_2O$; 0.18g $MnCl_2.\ 4H_2O$; 0.02g $ZnSO_4.7H_2O$; 0.01g $CoCl_2.6H_2O$; 0.009g $CuSO_4.5H_2O$; 0.006g $Na_2MoO_4.2H_2O$; 200mg Thiamine HCl; 1mg Biotin and 1mg Cyanocobalamin.

Experimental Design

Stock cultures were prepared by growing each microalgae strain in 250 ml conical flask containing 100 ml of the f/2 medium at 25°C for 4 days with orbital shaking at 150 rpm and illuminated with continuous light (NARVA LT 36W/077 Fluorescent lamp) at an intensity of 150 µmol photon m^{-2} s^{-1}. For experiment, each flask containing 450 ml autoclaved medium supplemented with filter sterilized various carbon sources (glucose, fructose, sucrose, lactose and galactose of 5 g each), were inoculated with 10% (v/v) of exponentially growing inoculums and then incubated at 25°C in an orbital shaker at 150 rpm in 12:12 hours light and dark up to 30 days. Photoautotrophic controls were maintained in each case.

Determination of Growth Rate

Growth rates were measured by spectrophotometer (Hitachi, U-2900) using 10mm quartz cuvette at 680nm wavelength (Lee *et al.,* 1998). Growth rate (K) was determined by calculating the difference in log of the final and initial optitcal density of biomass concentrations over time (Qin, 2005).

$$K = \frac{(\log ODf - \log ODi) \times 3.322}{T}$$

Where ODf is final biomass concentration, ODi is an initial biomass concentration and t is time in days. Generation time (G) in days can be obtained by using the formula,

$$G = \frac{0.301}{K.}$$

Estimation of Lipid and Protein Contents

Lipids were extracted with chloroform - methanol (2:1) following Pegg, (2001) protocol. Extraction of protein content was carried out by procedure described by Lowry (1951). Dubois *et al.*, 1956 protocol was followed for extraction and estimation of Total Carbohydrates. The total lipids, protein and carbohydrates were determined from a plot between yield and standards such as oleic acid, Bovine Serum Albumin (BSA), and D- glucose µg/ml and optical density was taken at 520, 595, 490nm respectively.

Statistical Analysis

All statistical analysis was done by using SPSS Version 21 software package. In this study the total lipid, protein and carbohydrate production from nutrient stressed microalgae strains were determined by the following equation

$$\text{Yield (mg/L)} = \frac{\text{Product value from standard curve}}{\text{Volume of digested material} \times \text{Culture volume}}$$

In our study, standards such as Cholesterol for lipid, BSA for protein and Glucose for carbohydrate with varying concentration were used for analysis. The hypothesis from our experiments and significance differences in the means were calculated by paired T test with p- value < 0.05 was considered as significant.

Result and Discussion

Effect of various organic carbon sources on Biomass productivity

Carbon is an essential for the growth of microalgae and is also required for the production of lipids. Growths of photoautrophic controls showed lowest cell density and growth rate (Fig. 1). Microalgae grown in glucose showed the highest culture density with fastest growth rate of 0.5065, 0.3122 and 0.2319 among other carbon sources (Table 1). Whereas the sucrose supplemented media showed the second highest growth rate and culture density. Slower growth rate and generation time of was observed in media supplemented with fructose.

Effect of organic carbon sources on lipid production

In the present study, the maximum concentration of lipid accumulation was observed in *Nannochloropsis salina* grown in glucose, 86.61 µg / ml in early stationary phase and 143.64 µg /ml in late exponential phase. Optical Density and statistical graph were shown in Figure 2a. It was found that the lipid content doubled in response to heterotrophic growth when compared to photoautotrophic growth. The sucrose also influences lipid accumulation as similar to glucose which are 143.35 µg/ml in late exponential phase. Compared to glucose and sucrose, fructose produces much less amount of lipids in cytosol. But the control produces only 115.93 µg/ml of lipid at 30 day of culture. In this study we found *Nannochloropsis salina* was the highest lipid producing microalgae than others.

In the case of glucose, the maximum concentration of lipids in *Dunaliella tertiolecta* was observed as 79.53 µg/ml and 123.57 µg/ml at 20th and 30th days respectively (Fig. 2b). It was higher when compared to cells grown under sucrose, fructose and control (113.29 µg/ml, 102.42 µg/ml and 104.09 µg/ml at 30th day). This species showed high lipid production in the order of glucose, sucrose, fructose, galactose and lactose treated culture. In *Tetraselmis suecica*, they produced 67.93 µg/ml and 123.83 µg/ml at 20th and 30th days

respectively (Fig. 2c) whereas 118.52 µg/ml lipids from sucrose. These findings indicate that the addition of glucose causes remarkable stimulation of lipid accumulation in microalgae. And also statistical analysis proved the glucose was best sugar to induce lipid when compare to other carbons and gave the significance < 0.01. Table 2 represents the overall metabolites production concentrations in the tested microalgae grown under different carbon sources.

Effect of Different carbon sources on protein production

The maximum concentration of protein were recorded in *Tetraselmis suecica* grown on glucose, we recorded 117.07 µg/ml of protein in 20th day and 84.11 µg/ml in late exponential phase (Fig 3a) because the proteins are essential component for cell growth and cell division, after entering in the exponential phase the marked decrease of protein concentrations were observed. Compared with fructose, Sucrose is much better to produce protein at moderate level (93.99 µg/ml at 20th day).

In the case of *Dunaliella tertiolecta*, the yield was slightly lower than *Tetraselmis suecica*. They synthesised proteins at concentration of 66.20, 89.48 and 68.07 µg/ml on 10th, 20th and 30th day respectively in media added with Glucose. In the case of control, fructose and sucrose showed less protein concentration. The variations in optical density and statistical data are shown in figure 3b.

Nannochloropsis salina was the microalgae which produce least protein among our experimented microalgae. The maximum protein concentration in *Nannochoropsis salina* was seen in glucose media, 61.52 µg/ml and 48.79 µg/ml on 20th and 30th days respectively (Fig. 3c). In our findings we suggest, the carbon sources showed higher differences in lipid production and accumulation but in the case of protein production they were very less when compared to nitrate limitation or nitrate stress.

Effect of Different carbon sources on Carbohydrate production

Nannochloropsis salina showed increased polysaccharide concentration of about 99.69 µg/ml, 125.83 µg/ml and 84.83 µg/ml on 10^{th}, 20^{th} and 30^{th} respectively grown on glucose. In sucrose they produced polysaccharides nearly 94.47 µg/ml, 113.78 µg/ml and 73.08 µg/ml on 10^{th}, 20^{th} and 30^{th} days respectively which is slightly higher than Control and fructose supplemented media (Fig. 4a). These results suggested the glucose was observed by microalgae faster than other sugars.

Total Polysaccharide concentration in *D. tertiolecta* was recorded in glucose media as 65.12 µg/ml, 93.77 µg/ml and 63.51 µg/ml on 10^{th}, 20^{th} and 30^{th} days respectively. In Sucrose 66.19 µg/ml, 92.50 µg/ml and 53 µg/ml on 10^{th}, 20^{th} and 30^{th} days whereas fructose showed 53.94 µg/ml, 74.32 µg/ml and 22.24 µg/ml on 10^{th}, 20^{th} and 30^{th} days respectively (Fig. 4b).

Tetraselmis suecica synthesised 64.58 µg/ml, 113.65 µg/ml and 82.52 µg/ml on 10^{th}, 20^{th} and 30^{th} days respectively. Figure 4c shows the variations in polysaccharides production. It was slightly higher than the algae grown on fructose and sucrose. This microalga produced polysaccharides slightly lower than *Nannochloropsis* and higher than *Dunaliella.* Overall biomolecule production in *N. salina, D. tertiolecta* and *T. suecica* are represented in Table 2.

Glucose stimulated rapid growth of the algae because it is simple sugar and can be easily assimilated to produce acetyl-CoA, which was then utilized in multiple pathways including the synthesis of fatty acids. Glucose and fructose had the same number of carbon numbers; they were decomposed by different enzymes. Glucose was converted into glucose-6-phosphate, which is key intermittent product involved in both glycolysis and pentose-phosphate cycle (Stewart, 1974). However, fructose could not be directly transformed to glucose-6-phosphate in microalgae. As a result, the growth rate was slightly decreased when fructose was used as the carbon source compared to glucose. Similarly, in our experiments all tested microalgae showed high lipid accumulation in the order of glucose, sucrose, fructose but fewer productions were seen in lactose and galactose. Higher rates of growth and respiration are obtained with glucose than with any other substrate, such as sugars, sugar alcohols, sugar phosphates, organic acids, and monohydric alcohols. Because glucose has much energy content per mol compared with other substrates. Glucose creates physiological changes and affects the metabolic pathways (carbon assimilation), cell size, storage compounds (starch and lipids grains) (Martinez *et al.*, 1991) protein, chlorophyll, RNA and vitamin contents in *Chlorella vulgaris*. *Chlorella, Tetraselmis* and *Nitzschia* showed increased growth rates under heterotrophic compared to autotrophic systems (Boyle and Morgan, 2009). Therefore heterotrophic cultivation of microalgae with different carbon source utilize simple, pure, and available carbon sources (glucose, fructose, sucrose, lactose and galactose) are most appropriate method to produce high lipid content in desired microalgae in large scale level for commercial uses. Therefore, the Biodiesel from microalgal lipid is an attractive, feasible alternative source because microalgae can significantly increase the production of lipids and their heterotrophic cultivation is now possible. For high lipid production, microalgae can be used as a best source for large scale production of biofuel in cheaper cost.

The present study shows the variations in data; it was compared and reported as, the selected 3 microalgae showed the increased protein content in log phase when compared to protein production in the stationary phase (Fig. 32, 33 and 34). Because of the metabolic shift occurred to produce the alternative metabolites such as lipids in stationary phase. It acts as storage compounds to maintain the cell survival ratio in the culture media.

A study was conducted on green microalgae *B. braunii* on effect of culture conditions on their growth, lipid and polysaccharide production with 16:8 hrs light dark cycles and continuous illumination. In continuous illuminations polysaccharide production reaches up to 1.6g/L (Dayananda *et al.*, 2007).

In general, the mixotrophic conditions and supplementation of organic carbon sources influence the biomass, lipid and carbohydrate concentrations but reduces the pigments and protein biosynthesis. Because nitrogen is an important source for the production of protein hence the increased carbon content alters the metabolic pathways and changes the biochemical compositions of the cells. In the present study it was proved that the addition of organic carbon source and optimization of light conditions gave impact on algal cell metabolism. We also obtain desirable products such as lipids, protein and carbohydrates in high level by switch over mechanism using stress.

The effects of various carbon substrates on growth, lipid and protein production by *N. salina, D. tertiolecta* and *T. suecica* were investigated with their respective controls. Glucose was found to be the best carbon source for cell growth and lipid accumulation when compared with microalgae grown under sucrose, fructose and photoautotrophic control. The highest lipid yield were observed in glucose supplemented cultures at stationary phase, 143.64 ± 6.15, 123.57 ± 5.55 and 123.83 ± 5.55 µg/ml for *N. salina, D. tertiolecta* and *T. suecica.* But the protein and carbohydrate content was slightly increased with the effect by organic sugar sources. The increased protein contents were observed in the order of *T. suecica, D. tertiolecta* and *N. salina* as 117.07 ± 3.65 µg/ml, 89.48 ± 3.25 µg/ml and 61.52 ± 3.06 µg/ml. Hence the *N. salina* grew on glucose has high potential for biodiesel production and *T. suecica* showed the best protein production among other microaglae.

Table.1 Growth rate and generation time of *N. salina, D. tertiolecta* and *T. suecica* grown under different carbon substrates

Sugars	Growth Rate (K)	Division Time (hrs)
N. salina		
Control	0.4185	17.25
Glucose	0.5065	14.26
Fructose	0.4515	15.99
Sucrose	0.4174	17.31
Lactose	0.4383	16.48
Galactose	0.4569	15.80
D.tertiolecta		
Control	0.2547	28.36
Glucose	0.3122	23.14
Fructose	0.2901	24.90
Sucrose	0.3046	23.71
Lactose	0.2374	30.42
Galactose	0.2573	28.07
T.suecica		
Control	0.1921	37.60
Glucose	0.2319	31.14
Fructose	0.2004	36.04
Sucrose	0.2005	36.02
Lactose	0.1953	36.99
Galactose	0.1877	38.47

Table.2 Metabolites (Lipid, Protein and Polysaccharides) production in *N. salina, D. tertiolecta* and *T. suecica* on heterotrophic conditions

Metabolites	Microalgae	Days	Control	Glucose	Fructose	Sucrose	Lactose	Galactose
Lipid	*N.salina*	10th Day	36.64 ± 4.58	41.52 ± 4.54	32.31 ± 4.63	35.37 ± 4.59	5.30 ± 5.08	6.13 ± 5.0635
		20th Day	72.38 ± 4.57	86.61 ± 4.74	73.40 ± 4.58	78.48 ± 4.63	47.23 ± 4.51	45.87 ± 4.52
		30th Day	115.93 ± 5.34	143.64 ± 6.15	115.06 ± 5.32	143.35 ± 6.14	73.70 ± 4.59	81.49 ± 4.67
	D.tertiolecta	10th Day	43.38 ± 4.53	40.43 ± 4.55	21.26 ± 4.77	33.28 ± 4.61	0.65 ± 5.18	10.62 ± 4.96
		20th Day	67.30 ± 4.54	79.53 ± 4.65	61.48 ± 4.51	78.39 ± 4.63	30.09 ± 4.65	40.03 ± 4.55
		30th Day	104.09 ± 5.06	123.57 ± 5.55	102.42 ± 5.03	113.29 ± 5.28	70.44 ± 4.56	83.08 ± 4.69
	T.suecica	10th Day	14.77 ± 4.89	38.17 ± 4.57	23.58 ± 4.74	34.53 ± 4.60	10.91 ± 4.96	15.94 ± 4.86
		20th Day	58.47 ± 4.50	67.93 ± 4.54	59.67 ± 4.50	59.11 ± 4.50	43.45 ± 4.53	44.81 ± 4.52
		30th Day	95.97 ± 4.90	123.83 ± 5.55	103.61 ± 5.05	118.52 ± 5.41	68.97 ± 4.55	74.47 ± 4.59
Protein	*N.salina*	10th Day	27.38 ± 3.18	40.93 ± 3.09	38.05 ± 3.10	57.07 ± 3.05	26.69 ± 3.19	34.35 ± 3.12
		20th Day	49.90 ± 3.06	61.52 ± 3.06	64.63 ± 3.07	62.60 ± 3.06	56.55 ± 3.05	56.48 ± 3.05
		30th Day	39.75 ± 3.09	48.79 ± 3.06	49.90 ± 3.06	46.01 ± 3.07	36.48 ± 3.11	37.00 ± 3.11
	D.tertiolecta	10th Day	27.90 ± 3.18	66.20 ± 3.07	53.93 ± 3.05	62.60 ± 3.06	36.94 ± 3.11	38.51 ± 3.10
		20th Day	56.81 ± 3.05	89.48 ± 3.25	79.72 ± 3.16	85.48 ± 3.21	65.71 ± 3.07	67.97 ± 3.08
		30th Day	51.87 ± 3.06	68.07 ± 3.08	53.90 ± 3.05	58.02 ± 3.06	61.13 ± 3.06	57.82 ± 3.06
	T.suecica	10th Day	38.44 ± 3.10	74.06 ± 3.11	57.30 ± 3.05	54.84 ± 3.05	39.56 ± 3.09	42.08 ± 3.08
		20th Day	66.04 ± 3.07	117.07 ± 3.65	83.81 ± 3.19	93.99 ± 3.30	73.09 ± 3.11	76.55 ± 3.13
		30th Day	59.89 ± 3.06	84.11 ± 3.19	56.22 ± 3.05	78.67 ± 3.15	53.21 ± 3.05	57.43 ± 3.05
Polysaccharides	*N.salina*	10th Day	55.783 ± 4.02	99.69 ± 4.44	64.55 ± 4.04	94.47 ± 4.35	54.74 ± 4.02	55.33 ± 4.02
		20th Day	92.06 ± 4.32	125.83 ± 5.02	114.39 ± 4.74	113.78 ± 4.73	73.25 ± 4.10	75.13 ± 4.11
		30th Day	51.93 ± 4.02	84.83 ± 4.22	84.20 ± 4.21	73.08 ± 4.09	51.29 ± 4.02	56.05 ± 4.02
	D.tertiolecta	10th Day	35.53 ± 4.11	65.12 ± 4.04	53.94 ± 4.02	66.19 ± 4.05	44.60 ± 4.05	43.06 ± 4.05
		20th Day	71.68 ± 4.08	93.77 ± 4.34	74.32 ± 4.10	92.50 ± 4.32	65.62 ± 4.05	60.73 ± 4.03
		30th Day	39.85 ± 4.07	63.51 ± 4.04	22.24 ± 4.25	53.00 ± 4.02	35.73 ± 4.10	39.38 ± 4.08
	T.suecica	10th Day	43.36 ± 4.05	64.58 ± 4.04	53.70 ± 4.02	65.02 ± 4.04	47.58 ± 4.03	51.36 ± 4.02
		20th Day	80.15 ± 4.16	113.65 ± 4.73	88.68 ± 4.27	94.44 ± 4.35	64.05 ± 4.04	63.95 ± 4.04
		30th Day	40.98 ± 4.07	82.52 ± 4.19	58.49 ± 4.02	51.46 ± 4.02	37.67 ± 4.09	40.45 ± 4.07

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