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Effect of Phosphate on Growth and Lipid (Tag) Production in Fresh Water Diatoms

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Abstract: ***The present work describes the effect of phosphate ($Ca_3(Po_4)_2$) on biodiesel production and growth of freshwater diatoms. The results revealed that the growth of diatoms in terms of number of cells was better at lower concentrations of phosphate .Maximum growth (1x104 cells/ml) could be recorded at 0.05 mg/l which was maintained up to 1.0 mg/l of phosphate concentration However, the growth of diatoms decreased Considerably with the increasing concentration of phosphate Beyond 1.0 mg/l. When the phosphate concentration was increased to 5.0 mg/l, the growth of diatoms was reduced drastically ($4x10^3$ cells /ml). The minimum growth (2 x 102) of diatoms in terms of number of cells was recorded in presence of 50 mg/l of phosphate. The addition of phosphate as a supplement was responsible for more chitin production which produced more biodiesel. It was also observed that the rate of biodiesel production was maximum at 900C.***

Keywords: Bio-diesel, diatoms, phosphate, TAGs, Growth

1. Introduction

Fresh water diatoms, belonging to the Division Chrysophyta, Class Bacillariophyceae are algae which form endoplasmic cysts, store oils rather than starch,possess a bipartite cell wall and secrete silica at some stage of their life cycle. Diatoms are commonly between 20-200 microns in diameter or length, although sometimes they can be up to 2 millimetres long (Carmichael, 1986).These are found in nearly every freshwater, marine aquatic habitat and soils, contributing to a large percentage of the global carbon budget through photosynthesis. The cell walls of diatoms contain polymerised phosphate, and they often accumulate oils and chrysolaminarin (McHugh, 2003). Microalgae are sunlight driven cell factories that convert carbon dioxide into Potential biofuels, foods, feeds and high value bioactives (Chisti, 2007). Microalgae can provide several different types of renewable biofuels which are produced by anaerobic digestion of the algal biomass. Biodiesel is derived from microalgal oil (Gavrilescu and Chisti, 2005) and photobiologically produced biohydrogen (Kalivani *et al.*, 2006). Biodiesel is currently being produced from plant and animal oils, mainly from soybeans on commercial scale. Canola oil animal fat, palm oil, corn oil, waste cooking oil (Felizardo *et al.*, 2006; Kulkarni and Dalai, 2006) and Jatropha oil (Barnwal and Sharma, 2005) are other sources of commercial biodiesel. Production of methyl esters (biodiesel) from microalgal oil has previously been demonstrated (Belarbi *et al.*, 2000). Although the product is intended for pharmaceutical use. Therefore, several companies are now attempting to commercialize production of biodiesel from Microalgae extracted oils used in making biodiesel consist of triglycerides (TAGs) in which three fatty acid molecules are esterified with a molecule of glycerol. In making biodiesel, triglycerides are reacted with methanol in a reaction known as transesterification or alcoholysis.Transesterification produces methyl esters of fatty acids that are biodiesel and glycerol. In a stepwise reaction triglycerides are first converted to diglycerides, then to monoglycerides and finally to glycerol. The present work was designed to evaluate the phosphate effect on production of lipid due to effect of phosphate concentration.

2. Materials and Methods

Collection and isolation of diatoms

Samples of fresh water diatoms were collected from Tones River (The Gangetic fresh water) on the Riwa road side which is 40 kilometer away from Allahabad, India. For site characterization by culturing, fresh water sub samples (10-50 cm deep) were taken from approximately 25 positions in each site of interest. These sub samples typically distributed along transects of interest were collected from random positions. The sub samples were combined in a sterile bag and homogenized by crushing and mixing. From each composite sample, one hundred-fold dilutions were made by adding 1g of homogenized sample to 99 ml of 0.7% saline solution. Agar media were poured in sterilized Petri dishes and aliquots of 0.1-10 ml were spread in agar-solidified (1.5% agar) Z-8 media (Carmichael, 1986) and 0.5 mg per liter of silicate was added for quantification of diatoms. Cultures were allowed to dry overnight before inversion, sealed with parafilm and incubated at 23-24°C under constant light until good growth was obtained.

Maintenance of Algal cultures

Freshwater diatoms cultures were grown under continuous light at an intensity of 400 to 500 foot candles .Cultures reached optimum growth in 7–14 days, after which the light intensity was reduced to 50 –100 foot candles. Diatoms cultures were transferred periodically to the fresh media. The well grown cultures can be stored for one to six months in an area with low illumination before sub culturing is required again (Charmichael, 1986).

Staining and cells counting

Diatoms abundance was obtained by settling the fixed samples in settling chambers, stained by Lugol'92s solution (Throndsen, 1978) and examined under an inverted microscope (Hasle, 1978; Gifford and Caron, 2000). Wet samples were smeared onto a slide for immediate examination and determination of possible further treatments. The sample was diluted and strewn onto cover slips, dried and mounted on slides. Because the refractive index of water and phosphate are very similar, a mounting medium with a higher refractive index was used in order to increase the contrast. Cells were counted using a haematocytometer for determination of growth of diatoms.

Estimation of total polysaccharide (chitin) content

The process of extraction involved deproteination with 2% w/v sodiumhydroxide solution (30:1 v/w, 90ºC, 2 hrs), Vijai Kumar Srivastava *et al.* separation of alkaline soluble fraction (AIF) by centrifugation (4000 rpm, 15 min), extraction of chitosan from AIF under reflux (10% v/v acetic acid 40:1 v/w,60ºC, 6 hrs), separation of crude chitin by centrifugation (4000 x g, 15 min) and precipitation of chitosan from the extract at pH 9.0, adjusted with a 4 M NaOH solution.Crude chitin and chitosan were washed on a coarse sintered-glass funnel with distilled water, ethanol and acetone and air-dried at 20ºC and weighed the extracted chitin (Franco *et al.*, 2004).

Estimation of total lipid (TAGs) content

TAGs extraction was done by double diffraction method. Dry biomass was mixed with petroleum ether (1:20 w/v) and centrifuged at 4000 rpm for 15-20 min.The supernatant was discarded and kept at room temperature for 24 hours. Two clear layers formed which was estimated gravimetrically as described elsewhere (Shahidi and Wanasundara, 1998).

Biodiesel production

The methyl esters (biodiesel) production was carried out according to Chisti (2007) involving the following steps.

a) Drying of Diatoms Biomass: After filtering the biomass was placed in a hot air ovan at 600C for drying.

b) Extraction and separation of Triacylglycerides (TAG) :

Dried biomass was placed in centrifuge tubes and mixed with petroleum ether (boiling range 60-800C, 1:20 (w/ v) diatom mass: petroleum ether) and centrifuged at 4000 rpm for 20 min in order to separate TAGs. The samples were kept at room temperature for 24 h. Two distinct layers were formed.

c) Transesterification of Triacylglycerides (TAG): TAG was collected using a pipette and mixed with three volumes of 80% methanol and catalyst potassium hydroxide (1%K OH) into a beaker and placed at 650C for 90 min in a hot air oven. Methyl ester and glycerol was obtained in3:1 ratio.

d) Biodiesel recovery: Biodiesel was purified by using partition chromatography. Methyl ester and glycerol mixture was placed in separating funnel along with distilled water. Since glycerol was soluble in distilled water hence two separate layers became visible. The upper layer was biodiesel and lower layer was glycerol. From 1.00 g of dry biomass 38 ml of methyl ester (biodiesel) and 20 ml of glycerol was obtained.

Table 1: Growth of diatoms in different medium of Phosphate concentrations.
Comparison of chitin against TAG content of diatoms

S.No.	Concentration of phosphate ($Ca_3(Po_4)_2$) in mg/litre	No of cell per ml (in 72 hours)
1	.12	$2x10^4$
2	1.10	$2x10^4$
3	2.20	$2x10^4$
4	10.5	$8x10^3$
5	20.80	$4x10^3$
6	100	$4x10^3$

3. Results and Discussion

Growth of diatoms

The growth was evaluated in terms of number of cells. It is apparent from table 1 that the growth of diatoms in terms of number of cells was better at lower concentrations of phosphate. Maximum growth ($2X\ 10^2$) could be recorded at concentration 0.01 mg/l which was maintained upto2.0mg/l of phosphate concentration. However, growth of diatoms decreased considerably with the increasing concentration of phosphate beyond 2.0 mg/l. When the phosphate concentration was increased to 10.0 mg/l, the growth of diatoms was reduced drastically ($8x10^3$). The minimum growth of diatoms in terms of number of cells was recorded in presence of 100 mg/l of phosphate. Growth inhibition at higher concentration of phosphate has also been reported by Alverson (2007). When phosphate was used up in excess amount, the cell division possibly slowed down, since phosphate is a component of the diatoms cell walls. Blocking of pore of frustules at 50mg/l concentration phosphate may be another reason for inhibition of growth. Thus, phosphate plays an important role in the growth of diatoms up to some extent. In 1986, researchers at NREL reported on the use of phosphate depletion as a way to increase oil levels in diatoms. In the diatoms *Cunninghamella cryptic*, the rate of oil production remained constant once phosphate depletion occurred, while growth rate of the cell dropped. Further studied identified two factors that seemed to play role in this species; (1) phosphate depleted cells direct newly assimilated carbon more towards lipid production and less towards carbohydrate production. (2) phosphate depleted cells slowly convert non-lipid cell components to lipids.

Total polysaccharide (chitin) content was extracted from diatoms treated with different concentrations of phosphate ($Ca_3(Po_4)_2$) as a supplement in growth medium. It was found that the chitin content (270 mg/g) increased with the increasing concentration of phosphate (fig.1).

Conclusively, the addition of phosphate as a supplement was responsible for more chitin production and it converted in fats and produced more biodiesel.

Biodiesel production

A time limiting reaction (transesterification) for the production of biodiesel at different temperatures that varies between 60 to 950C were done and it was observed that the

rate of biodiesel production was increased up to 900C and above that temperature it remained constant (table 2). Transesterification requires 3 mol of alcohol for each mole of triglyceride to produce 1 mol of glycerol and 3mol of methyl esters while industrial processes use 6 mol of methanol for each mole of triglyceride. This large excess of methanol ensures that the reaction is driven in the direction of methyl esters, i.e. towards biodiesel. Yield of methyl esters exceeds 98% on a weight basis (Fukuda *et al.*, 2001). Transesterification is catalyzed by acids, alkalis (Fukuda *et al.*, 2001; Meher *et al.*, 2006) and lipase enzymes (Sharma *et al.*, 2006). Alkali-catalyzedtransesterification is about 4000 times faster than the acid catalyzed reaction (Fukuda *et al.*, 2001). Consequently, alkalis such as sodium and potassium hydroxide are commonly used as commercial catalysts at a concentration of about 1% by weight of oil. Alkoxides such as sodium methoxide are even better catalysts than sodium hydroxide and are being increasingly used. Use of lipases offers important advantages, but is not currently feasible because of the relatively high cost of the catalyst (Fukuda *et al*., 2001). Alkali catalyzed transesterification is carried out at approximately 60°C under atmospheric pressure, as methanol boils off at 65°C at atmospheric pressure. Under these conditions, it takes about 90 min to complete the reaction. A higher temperature can be used in combination with higher pressure, but this is expensive. Methanol and oil do not mix; hence the reaction mixture contains two liquid phases. Other alcohols canbe used, but methanol is the least expensive.

4. Summary and Conclusion

It is estimated that diatoms account for up to 25% of the total oxygen production on earth suggesting it to be enormously successful organism. The main storage compounds of diatoms are lipids (TAGs) and a 1, 4-linked carbohydrate known as chrysolaminarin. From the above experiment it may be concluded that, microalgal biodiesel is technically feasible. It is the only renewable biodiesel that can potentially completely replace liquid fuels derived from petroleum. Economics of producing microalgal biodiesel need to be improved substantially to make it competitive with petro diesel. Producing low-cost microalgal biodiesel requires improvements to algal biology through genetic and metabolic engineering. From the above results it may be concluded that 0.05 mg per liter phosphate concentration promoted the TAGs biosynthesis and consequently resulted in higher production of methyl ester (Biodiesel) and that the fresh water diatom can be used at industrial level for the production of methyl ester (Biodiesel).

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