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Effect of Various Nitrogen Sources on Microalgal Growth and Lipid Content in *Chlorella pyrenoidosa* NCIM 2738 and ANK-1

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A B S T R A C T

The rapid population growth, fast agricultural extensions, increased urbanization and industrialization have together stressed the exploration of eco-friendly sustainable and renewable energy resources in meeting the energy needs. Biodiesel is a fuel that comprises mono-alkyl esters of long-chain of fatty acids derived from vegetable oils. Microalgae have tremendous capacity of biosynthesis of TAG compared to other terrestrial crops. The aim of present work was to study effect of various nitrogen sources on the patterns of microalgae growth and the lipid content. The two cultures selected were *Chlorella pyrenoidosa* NCIM 2738 recognized culture by NCIM, and ANK-1 a culture collected from Ankleshwar in Gujarat. According to morphological study under microscope ANK-1 was identified as *Chlorella sp.* various nitrogen sources used were ammonium sulphate, urea and potassium nitrite in BG-11 and Bolds and Basal medium. The objective is to monitor the change in the growth pattern subsequently the lipid content under different medium. The study confirms the impact on growth and lipid content while changing the source of nitrogen wherein; BG-11 medium containing nitrogen sources like ammonium sulphate, urea and potassium nitrite resulted in good growth in *Chlorella pyrenoidosa* whereas, the medium containing urea proved good in case of ANK-1.

Introduction

The microalgae are unicellular photosynthetic organisms that use light energy and carbon dioxide, with higher photosynthetic efficiency than plants for the production of biomass (Benemann 1997, Miao, Wu, 2006). Microalgal lipids are regarded as the feedstock of future sustainable biodiesel production owing to much higher growth rates and photosynthetic efficiencies than conventional terrestrial plants (Chisti 2007; Li *et al.,* 2008; Liu *et al.,* 2008; Usui and Ikenouchi 1997). They may be destined to different applications, as biofuel production, purification of wastewater either autotrophic or mixotrophic conditions (Orús, *et al.*, 1991, Mu˜noz, *et al.,* 2006) extractions of high added value foods and pharmaceutical products, or as food for aquaculture (Spolaore, *et al.,* 2006). The energy crisis has now become one of the biggest challenges of the 21st century. Oil and natural gas storage all over the globe have been estimated to be depleted in 40 and 64 years, respectively (Vasudevan and Briggs, 2008). Microalgae biomass contains approximately 50% of carbon on a dry weight basis (Sánchez Mirón *et al.,* 2003) and all the carbon present in cell usually from carbon dioxide. With the production of 100 tons of microalgae biomass, around 180 tons of CO_2 can be

disposed using natural or artificial light making the entire application as eco-friendly behavior. The lipids from microalgae could be used in different processes for energy exploitation, including the simple combustion in boiler or in a diesel engine. However, the best possible use of this oil is certainly its transformation to a biofuel, especially biodiesel (Chisti, 2007). Moreover, high added value compounds can be extracted from microalgae, such as fatty acids (linolenic, arachidonic, eicosapentaenoic, docosahexaenoic acids, etc.) (Cardozo, *et al.*, 2007 and Valencia *et al.,* 2007) pigments (carotenoids and ficobiliproteins), biochemically stable isotopes (Chisti 2007 and Borowitzka, *et al.,* 1991) and vitamins such as biotin (Baker *et al.*, 1981) vitamins C (S.A. Survase *et al.,* 2006) and vitamin E (Running *et al.,* 2002 and C. Bremus *et al.*, 2006) besides some metabolites appear to have some pharmacological activities, among others the anticholesterolemic, antitumoral, immune modulatory, antibacterial and antimycotic ones. The biomass productivity, lipid cell content, and overall lipid productivity are some of the key parameters affecting the economic feasibility of algal oil for biodiesel production and associated buy products. Several studies have shown that the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions temperature and light intensity or nutrient media characteristics concentration of nitrogen, phosphates and iron (IIIman *et al.*, 2000 and Liu, 2008).

The lipids extracted from the two microalgae grown in Erlenmeyer flasks under conditions favoring lipid content in the cell have been analyzed quantitatively and qualitatively, by gravimetric and gas chromatographic methods, and different extraction methods have been investigated to maximize the yield in biodiesel. Nutrient limitation is an efficient environmental pressure to increase the lipid accumulation (Goldberg and Cohen, 2006 and Rodolfi *et al.,* 2009). For instance, nitrogen limitation would cause three Changes; decreasing of the cellular content of thylakoid membrane, activation of acyl hydrolase and stimulation of the phospholipid hydrolysis. These changes may increase the intracellular content of fatty acid acyl-CoA. Meanwhile, nitrogen limitation could activate diacylglycerol acyltransferase, which converts acyl-CoA to triglyceride (TAG) (Takagi *et al.,* 2000). Therefore nitrogen limitation could both increase lipid and TAG content in micro-algal cells. Many factors affect these properties, such as nutrient concentration (Aslan and Kapdan, 2006 and Goldberg and Cohen, 2006; Rodolfi *et al.,* 2009), CO2 aeration (Chiu *et al.,* 2009), light conditions (Solovchenko *et al.,* 2008), and so on. The concentration of nitrogen and phosphorus present in water is considered to be the fundamental factor and has a direct influence on algal growth kinetics, which relates to nutrient removal and lipid accumulation closely. In present study was conducted to investigate the growth response and lipid content of a fresh water green algae NCIM 2738 *Chlorella pyrenoidosa* and ANK-1, by varying the nitrogen sources in different growth medium.

Materials and Methods

Organism and growth conditions

The culture of green alga *Chlorella pyrenoidosa* NCIM 2738 was provided by NCIM, India. The ANK-1 culture was collected from fresh water pond at Ankleshwar, Gujarat, India. These cultures were grown in 250 ml Erlenmeyer flasks with 150ml medium. The medium used for cultivation was BBM and BG-11. The nitrogen source used was through the medium as urea, ammonium sulphate and potassium nitrate. The cultures were performed at

thermally controlled algal laboratory at 25° C along with 16 hour fluorescent illumination (white tube light). The cultures were routinely hand shaken two to three times daily to avoid sticking. All the glassware's and media were sterilized prior to inoculation.

Cell growth and biomass analysis

Optical density measurement at 660 nm was used to monitor cell growth by UV/visible spectrophotometer with every three day time interval for 21 days. The total biomass weight was taken gravimetrically after 21 days. The biomass is harvested by centrifugation at 3000 rpm for 10 minutes in 15ml Eppendorf tubes. The algal sample is kept in dried and pre-weighed crucibles. After drying sample crucibles were again weighed to know the total biomass of algae.

Total biomass = C2 – C1

Where,

C2 is crucible with dried algae
C1 is empty pre-weighed crucible

Lipid extraction

Microwave Digestion

Digestion is the process in which the algal cell wall is denatured and the total lipid comes out. In microwave digestion method by using Microwave Digestive system the algal cell is digested. Pre-weighed dry powdered algal mass is taken in Teflon vessels along with 6 ml of distilled water, in thoroughly washed Teflon vessels.

Teflon vessels are sealed with lids and loaded into the digestive system. These vessels are subjected to 175^0 C and 40 bar pressure. Time for 1 Cycle is taken as: 26minutes.

Lipid extraction from the Digested algal biomass was done using the method of Bligh and Dyer (1959) with some modifications. 2 ml methanol and 1 ml chloroform was added to the dried algal biomass. The mixture was agitated on vortex for 2 minutes. The layers of the mixture were separated by centrifugation at 2000 rpm for 10min. The supernatants collected and evaporation was carried out on heating mantle at 80° C till constant dryness was achieved.

Lipid content
(% dry cell weight) = W2 - W1 × 100

Where,

W1 is the weight of empty silica crucible and
W2 is the weight of crucible + lipid

Results and Discussion

The time course biomass profile of *Chlorella pyrenoidosa* NCIM 2738 *and ANK-1* grown autotrophically in BG-11 and BBM medium with different nitrogen sources (urea, ammonium sulphate and potassium nitrate) in the medium both in exponential and stationary phase. The original nitrogen source concentration in the medium was 1 g/L. Different nitrogen sources were used to investigate the effect on algal growth. Growth of algae increases from the 1^{st} day starting from log phase to stationary phase then decline phase till 24 days. Figure-1 explains that in nitrogen source as ammonium sulphate the ANK-1 shows long log phase while the *Chlorella pyrenoidosa* NCIM 2738 shows short log phase and long stationary phase. Figure- 2 shows that in urea as nitrogen source both *Chlorella pyrenoidosa* NCIM 2738 *and ANK-1* show long log phase then stationary phase.Figure-3 highlights that in nitrogen source as potassium nitrate *Chlorella pyrenoidosa* NCIM 2738 *and ANK-1* are showing the long log phase.

The results interpret that the ammonium sulphate containing BG-11 the NCIM 2738 *Chlorella pyrenoidosa* shows higher optical density i.e. 1.70 while the ANK-1 shows optical density 0.60 after 21 days of incubation. The BG-11 that contains urea as nitrogen source brings out the information that the NCIM 2738 *Chlorella pyrenoidosa* mark with high growth bearing 0.65 optical density while ANK-1 growth is marked with the optical density as 0.46 after 21 days of incubation. Whereas, potassium nitrate containing BG-11 medium NCIM 2738 *Chlorella pyrenoidosa* shows fast growth rate with 0.39 optical density after 21 days of incubation while ANK-1 has 0.35 optical density with slower growth rate.

Figure. 4 explains that in nitrogen source as ammonium sulphate in BBM medium the *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 both are in log phase but after some interval of time the growth of *Chlorella pyrenoidosa* NCIM 2738 increases over the growth of ANK-1. Figure. 5 shows that in nitrogen source urea *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 both are in log phase. Figure. 6 interprets that in nitrogen source potassium nitrate the *Chlorella pyrenoidosa* NCIM 2738 show good growth initially in log phase and then enters stationary phase while ANK-1 is marked with very slow growth.

In case of BBM medium containing different nitrogen source the *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 the *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 have shown relatively same growth in ammonium sulphate contains BB medium with 0.21 and 0.27 optical density.

In case of urea both the sample cultures have shown relatively same growth but higher than ammonium sulphate i.e. O.D. is 0.79 and 0.78 respectively. Whereas in BB medium containing potassium nitrate as nitrogen source there is huge difference in growth i.e. optical density after ANK-1 has 0.37.

Fig.1 Growth curve of *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 grown on BG-11 medium with ammonium sulphate as nitrogen source

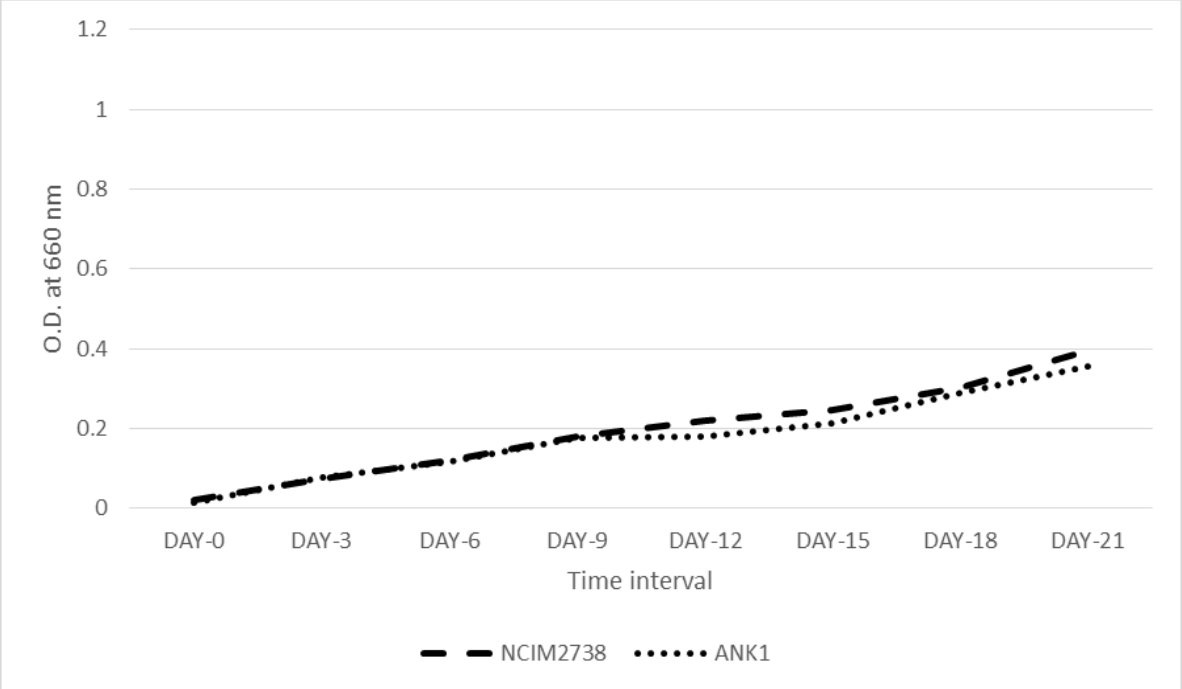

Fig.2 Growth curve of *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 grown on BG-11 medium with urea as nitrogen source

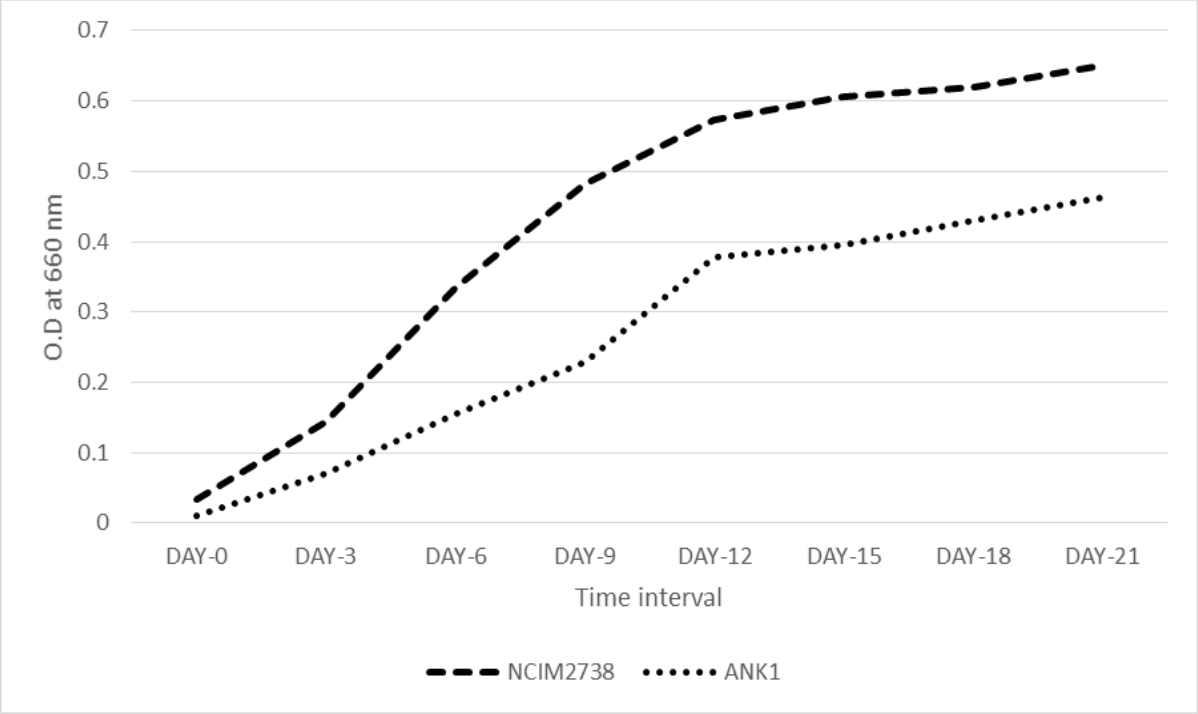

Fig.3 Growth curve of *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 grown on BG-11 medium with potassium nitrate as nitrogen source

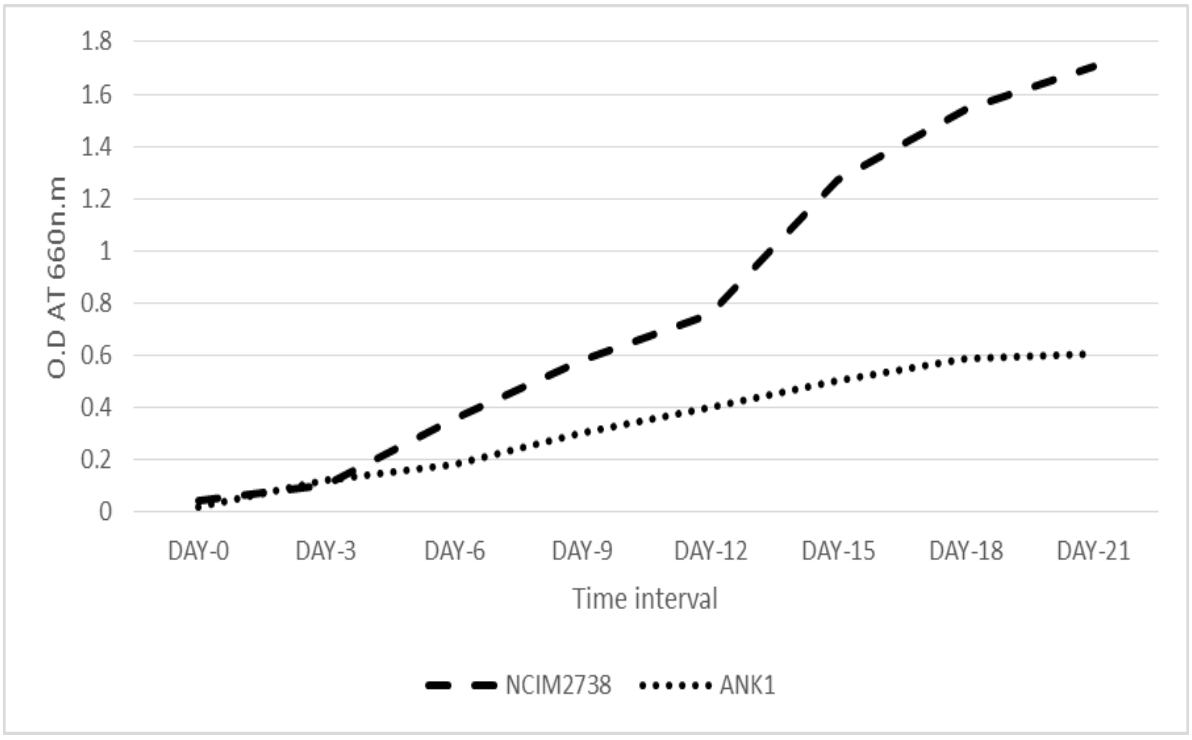

Fig.4 Growth curve of *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 grown on BB medium with ammonium sulphate as nitrogen source

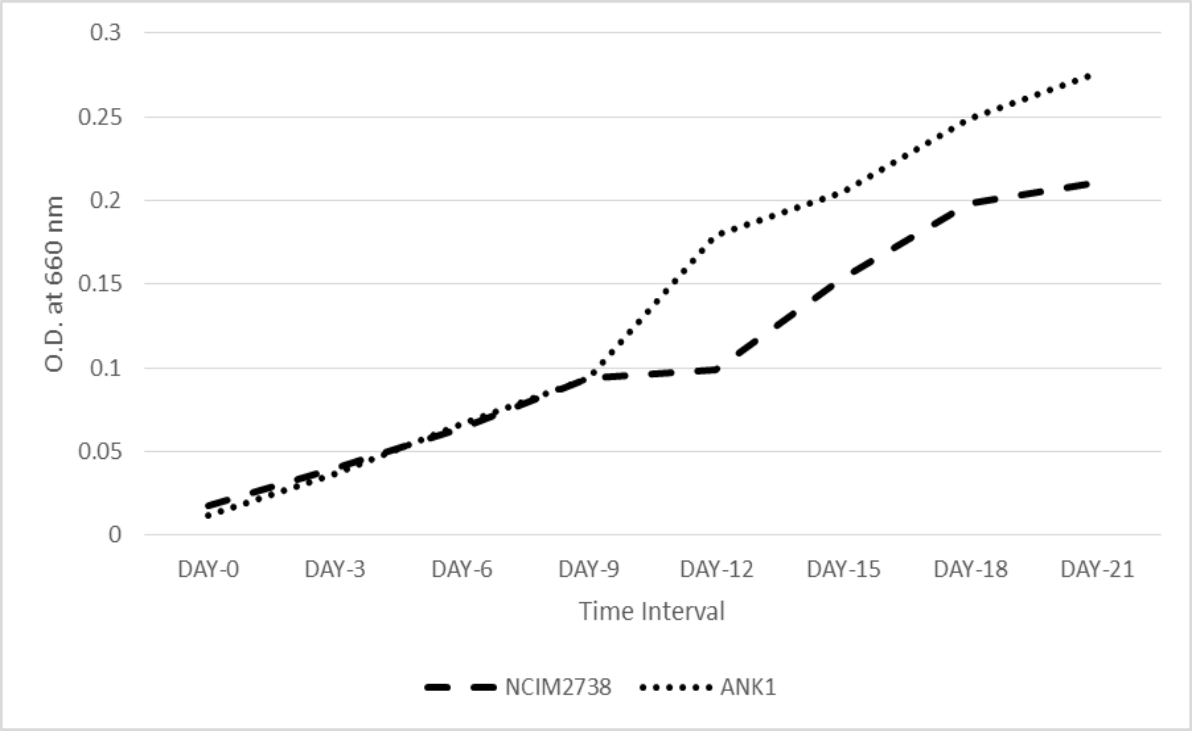

Fig.5 Growth curve of *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 grown on BB medium with urea as nitrogen source

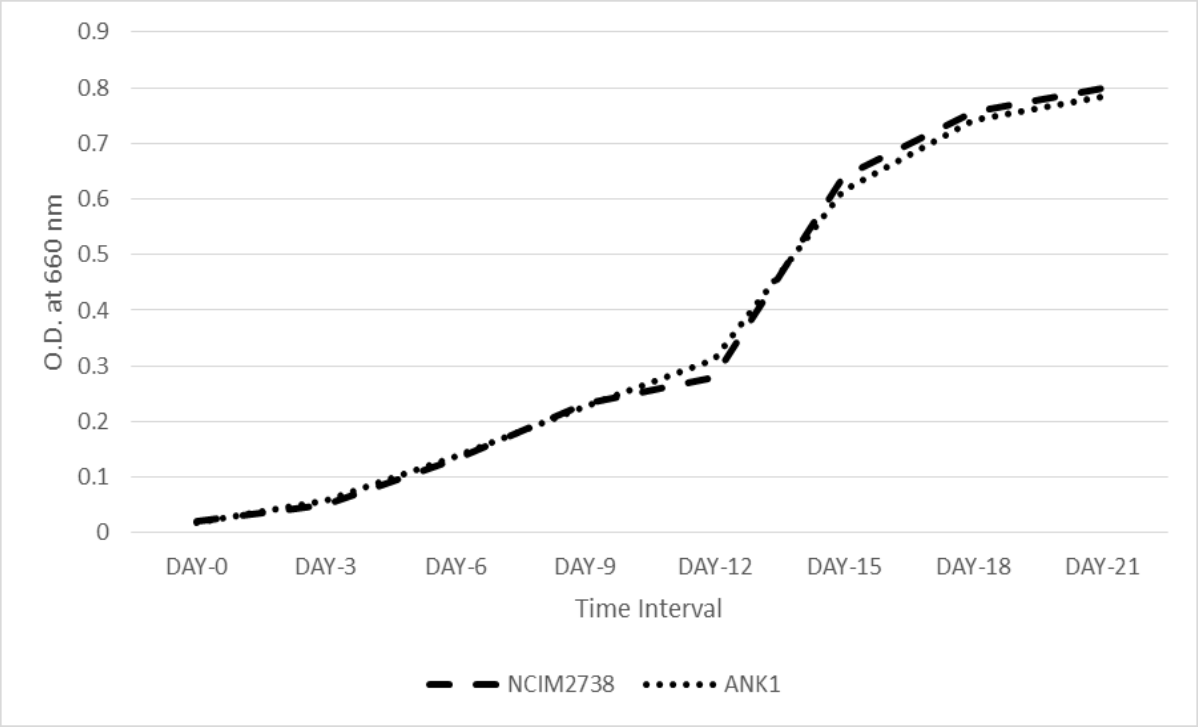

Fig.6 Growth curve of *Chlorella pyrenoidosa* NCIM 2738 and ANK-1 grown on BB medium with potassium nitrate as nitrogen source

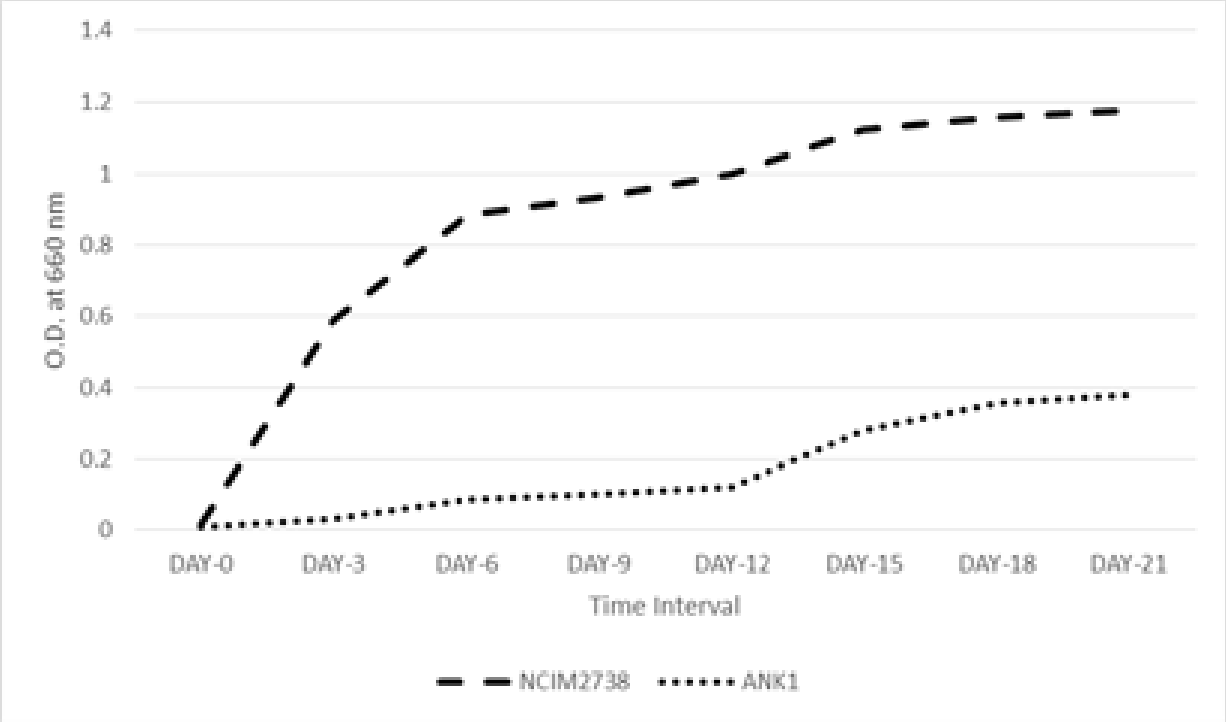

Fig.7 Comparison of lipid content of *Chlorella Pyrenoidosa* NCIM 2738 and ANK-1 grown in BG-11 medium with different nitrogen sources

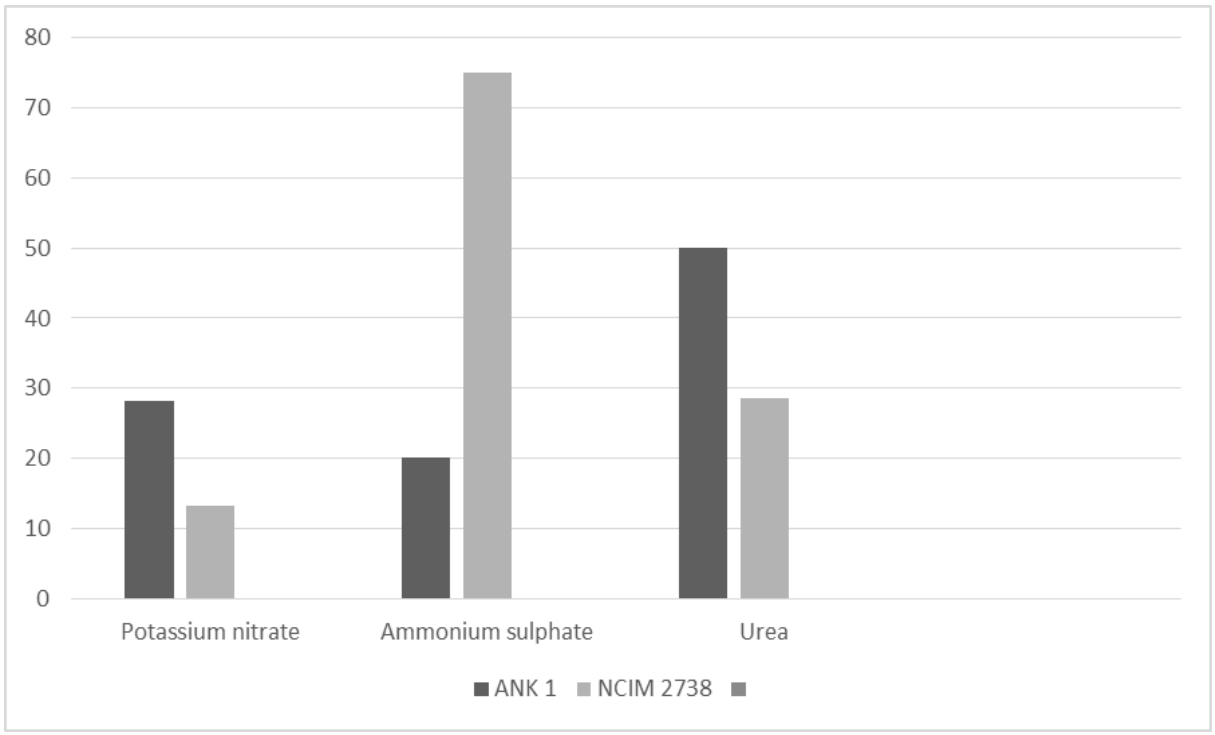

Fig.8 Comparison of lipid content of *Chlorella Pyrenoidosa* NCIM 2738 and ANK-1 grown in BB medium with different nitrogen sources

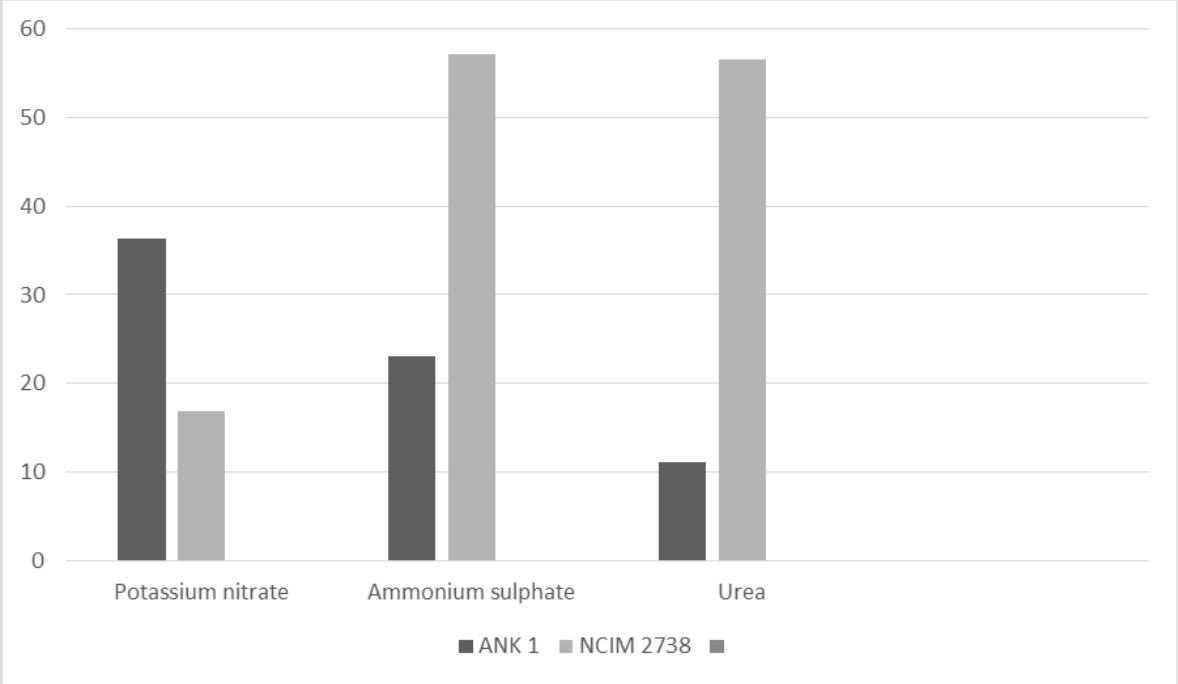

Table.1 Comparison of total biomass of in different nitrogen sources

BG-11	NCIM 2738	ANK-1
Urea	0.028 gm	0.01 gm
Potassium Nitrate	0.076 gm	0.032 gm
Ammonium Sulphate	0.008 gm	0.03 gm

Table.2 Comparison of total biomass of in different nitrogen sources

BBM	NCIM 2738	ANK-1
Urea	0.023 gm	0.108 gm
Potassium Nitrate	0.071 gm	0.022 gm
Ammonium Sulphate	0.007 gm	0.013 gm

Figure 7 shows the lipid content of *Chlorella pyrenoidosa* NCIM 2738 in different nitrogen sources. wherein *Chlorella pyrenoidosa* NCIM 2738 show higher lipid in ammonium sulphate while ANK-1 show higher lipid in urea as nitrogen source in BG-11 medium. Figure. 8 highlights the BBM medium containing ammonium sulphate and urea *Chlorella pyrenoidosa* NCIM 2738 showing higher lipid content while the ANK-1 shows higher lipid in BBM medium containing potassium nitrate. In different organic and inorganic nitrogen source in BBM medium the lipid accumulation is higher in *Chlorella pyrenoidosa* NCIM 2738 ammonium sulphate i.e. 57.14 %. In BBM medium the lipid accumulation is higher in ANK-1 containing potassium nitrite as nitrogen source i.e. 36.36 %. In different organic and inorganic nitrogen source in BG-11 medium the lipid accumulation is higher in *Chlorella pyrenoidosa* NCIM 2738 ammonium sulphate i.e. 75.00 % while in ANK-1 higher lipid is found in urea i.e. 50.00 %.

Biofuel can be produced economically and sustainably from micro-algae. Both biomass and lipid play an important role in biofuel production. Various studies are going on to enhance the algal growth and lipid content for biofuel production. Different nitrogen sources influence algal growth and lipid content differently i.e. *Chlorella pyrenoidosa* NCIM 2738 is showing good growth in urea and potassium nitrate as nitrogen source in BG-11 medium. ANK-1 is showing good growth in ammonium sulphate and urea as nitrogen source in BB medium, while showing little growth in potassium nitrate.

In conclusion, the present study suggests that most effective way to enhance microalgal growth and lipid is to provide proper nitrogen source. The most effective nitrogen source for *Chlorella pyrenoidosa* NCIM 2738 is urea and potassium nitrate while for ANK-1 the suitable nitrogen source is urea for growth. *Chlorella pyrenoidosa* NCIM 2738 show high lipid content in nitrogen source ammonium sulphate and urea whereas ANK-1 show high lipid in potassium nitrate and urea. However, if the observation time is extended the growth rate both in terms of biomass and lipid content will definitely be better than what has been observed during the course of this study.

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