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Impact of different nitrogen sources on biomass growth and lipid productivity of *Scenedesmus sp.* for biodiesel production

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Abstract
Microalgae can accumulate considerable amounts of lipids under different nutrient deficient conditions, making them as one of the most promising sustainable sources for biodiesel production. Nitrogen deprivation is one of the common stresses encountered by microalgae in nature and it is reported that under nitrogen deprived state biomass and lipid productivity get enhanced. In present investigation a microalga isolated from fresh water lake Bhimtal, India, was identified as *Scenedesmus sp.,* DBTKU. Isolate was also examined for biomass and lipid content in BG-11medium containing different concentrations of nitrogen sources viz. sodium nitrate, potassium nitrate and ammonium nitrate. It was observed that *Scenedesmus sp.,* was able to utilize all examined sources of nitrogen at different concentrations. Our results reveal that nitrogen stress typically has disproportionate effects on promoting biomass growth and lipid content. Sodium nitrate was found to be the most appropriate nitrogen source of biomass growth and lipid content. It was observed that at 1.0 gm/l sodium nitrate, isolate showed better biomass growth and lipid productivity. Potassium nitrate (1.5gm/l) also showed almost similar affect on biomass growth and lipid content but ammonium nitrate exhibited moderate effect on biomass growth and lipid content. We observed that higher nitrogen beyond (2.0gm/l) was not much responsive for higher lipid content as compared to lower concentration invariably for all the tested nitrogen sources.

Key words. Microalgae, *Scenedesmus sp.,* Biomass*,* Lipid productivity, Biodiesel

Introduction:
The perspective of global ecosystem, life as we know, it depends on energy. There is no denying the fact that energy is one of the most essential and vital component for development and global economy literally runs on it (Zhang *et al.,* 2011). Energy use pattern of today is directly linked with economic prosperity, development and quality of life. So the need of energy is increasing continuously because of increase in industrialization and population. Currently, the three non renewable energy sources petroleum, coal and natural gas are providing 90% of energy globally (Kulkarni and Dalai, 2006; Dale 2008). The growth projections estimate a nearly 50% increase in transport energy use by 2030 and CO_2 emissions more than 80% by 2050. The world population has grown from 2 billion to 7 billion from the second world war to 21st century (Avni and Blazquez, 2011). Presently the world is witnessing increasing energy crisis is also representing the major challenge of this century (Vasudevan and Briggs, 2008). Fossil fuel consumption, increasing cost, reliance on imported fuel, negative environmental impact and politically hostile regimes has led to an urgent need to develop alternative fuels. Heightened global awareness about global warming and consumption of finite fuel sources has driven research in biomass-based energy production. Biomass has always been a reliable source of energy. While last few decades it has seen that lignocellulosic biomass was used as a source of biofuel, but it was also plagued by lower yields, competes with arable land, causes deforestation and production cost closely resembles the price of fossil fuel in open market (Bisht *et al.,* 2016). Given the expanding market, surprisingly the researchers, entrepreneurs and investors view this as a significant opportunity to develop novel biological approaches to the production of biofuel from photosynthetic microbes such as microalgae and cyanobacteria. In this concern, both the unicellular microalgae and cyanobacteria (blue green algae) are at the forefront of research efforts aimed at developing technologies with relevant working model for the production of biofuel (Bhatt *et al.,* 2014). Among the major microorganisms utilized for biofuel extraction, microalgae represent an exceptionally diverse, highly specialized group adapted to various ecological habitats acting as primary producer in the water bodies and these water bodies are cover more than 71% of the earth space (Bisht *et al.,* 2015). These are relatively more efficient in converting sunlight into chemical energy due to their simpler cellular structure and efficiently produce cellulose, starch, oils in large amounts. A wide range of oil content (4-80%) of microalgal biomass has shown 250 fold higher biodiesel productions as compared to other sources of biodiesel production (Spolaore, *et al.,* 2006; Chisti, 2007). A variety of culture conditions such as sulfur **(**Matthew *et al.,* 2008**)**, nitrogen (Wang *et al.,* 2009; Work *et al.,* 2010), phosphorus (Weers *et al.,* 1997), zinc, or iron deficiency (Kropat *et al.,* 2011) or other growth inhibiting stressors such as high salt

(Siaut *et al.,* 2011) or high light (Zhekisheva *et al.,* 2002) were found to have a profound effect in causing a significant enhancement in the production of lipid in these organisms. Among these factors, nitrogen is known to have a strong influence on biomass growth and metabolism of lipids and fatty acids in various microalgae (Griffiths and Harrison, 2009). Microalgal oils can be converted to diesel, gasoline and jet fuel using existing technology (Chisti, 2007; Demirbas, 2011). Many microalgae species can be induced to accumulate substantial quantities of lipids thus contributing to a high oil yield (Sheehan *et al.,* 1998).

Material and method:

Isolation of *Scenedesmus sp.* Culture

For isolation of microalgae culture, serial dilution method was applied. Agar plates will be prepared by dissolving 2% agar (w/v) in BG-11 medium. After the spreading of sample in axenic condition, keep them in incubation at least seven days to grow in algal culture lab at 22±2°C temperature, 6470 lux light intensity operated on 12:12 hour's photoperiod (Ilavarasi *et al.,* 2012). No special provisions were made for CO_2 supply. After the incubation of seven days different type of microalgae colony were observed. Well single pure colony was picked up than again re-streaked to fresh BG-11 agar medium and incubated by similar way. The process was repeated until cultures were made single colony type. After growth, different pure colonies were inoculated in different Erlenmeyer flasks containing 70 ml of BG-11 broth medium and incubated in a by similar way with three times manual shaking per day.

Morphological characterization

Different single type of microalgae cultures were examined morphologically in a light microscope for preliminary identification and confirmation of the cultures was using inverted microscope (400 x magnifications, LEICA DMIL LED fluo). Preliminary identification of the algal cultures was made using a field guide (Prescott, 1978) and comparing with earlier reported species (http://www.algaeweb.net).

Molecular Characterization:

Genomic DNA was extracted from microalgae. The ITS2 rDNA was amplified by PCR with primer: forward 5'-GAGCATGTCTGCCTCAGC-3' and reverse 5'- GGTAGCCTTGCCTGAGC 3'. PCR product was sequenced and sequence was BLAST in NCBI database.

Growth and biomass estimation on synthetic BG-11 medium:

All microalgae isolates were assessed for the ability to grow at 22±2 °C on BG-11 medium. Equal amount of the inoculums of each isolates were inoculated in medium at the beginning of the experiment and cell viability was also compared. Growth was quantified at regular intervals of three days by measuring the optical density (OD) at 540nm using spectrophotometer and extending up to 15 days. Microalgae sample were shaked to prevent the settling to avoid erroneous reading while taking the OD. Growth and biomass productivity of microalgae was determined by measuring wet and dry biomass on the final day of experiment. The samples were centrifuged at 10000 rpm for 10 min then oven dried at a temperature of 70±2°C. Dried cell mass was measured and expressed in gram per liter (g/l).

Lipid estimation

Lipid extraction was done by following the protocol of Bligh and Dyer (1959) with minor modification. For 100 mg of dried microalgal biomass, 2 ml of methanol and 1 ml of chloroform was added and kept for 18 hours at 25°C. The mixture was agitated in vortex for 2 min. 1 ml of chloroform was again added and the mixture was shaken vigorously for 1 min. After that, 1ml of distilled water was added and the mixture was mixed in a vortex again for 2 min. Three layers were separated by centrifugation for 10 min at 5000 rpm. The lower layer was separated and the procedure was again repeated with the pellet. The two supernatants collected were allowed to stand for 2 h. Evaporation was carried out in hot air oven at 80°C for 50 min.

Experimentation:

The experiment carried out with three different nitrogen sources viz., potassium nitrate, sodium nitrate and ammonium nitrate. Four different concentrations of each nitrogen source was used, control (without

nitrogen), 0.5gm/l, 1.0gm/l, 1.5gm/l, and 2.0 gm/l added to supplement the basal BG-11 medium (devoid of nitrogen source). The media was prepared by mixing the different constituents at an appropriate amount.

Result:

Morphological characterization:

Sample obtained from Bhimtal Lake and allowed to grow in laboratory conditions. Morphological feature of the isolate have demonstrated its close similarity with genus *Scenedesmus sp.* Cells are green color, unicellular, spherical in shape its shows the **figure 1**.

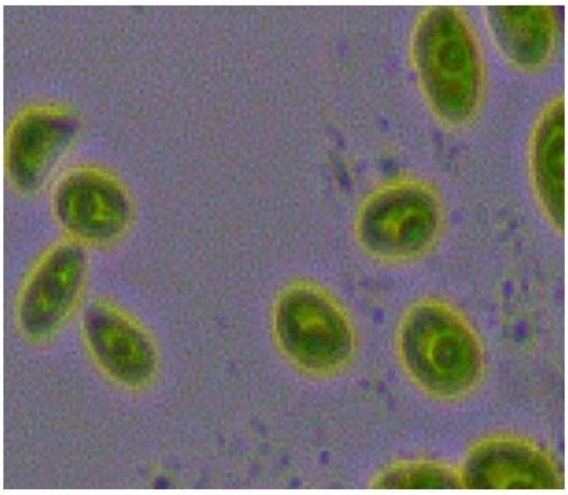


Fig 1: Morphological image of *Scenedesmus sp.*

Molecular characterization:

Amplified ITS2 rDNA sequence was sequenced and BLAST on NCBI. The BLAST analysis showed the highest percentage identity of 100% and quary cover of 100% with *Scenedesmus sp.* The sequence of isolate have been deposited into the National Center for Biotechnology Information (NCBI) under accession numbers of KT581427.

Effect on biomass growth and lipid yield

The growth data obtained for three different nitrogen sources over 15 day of incubation. Four different concentrations of each nitrogen source were used to supplement the basal BG-11 medium. The media was prepared by mixing the different constituents at an appropriate amount.

Effect of Sodium nitrate on biomass growth

The effect of sodium nitrate on growth and biomass yield of *Scenedesmus sp.* was examined. Highest growth and biomass yield (2.53±0.03 gm/l) was recorded at 1.0 gm/l sodium nitrate concentration where as 1.5gm/l concentration of sodium nitrate showed 2.36±0.02 gm/l biomass yield in **figure 2**.

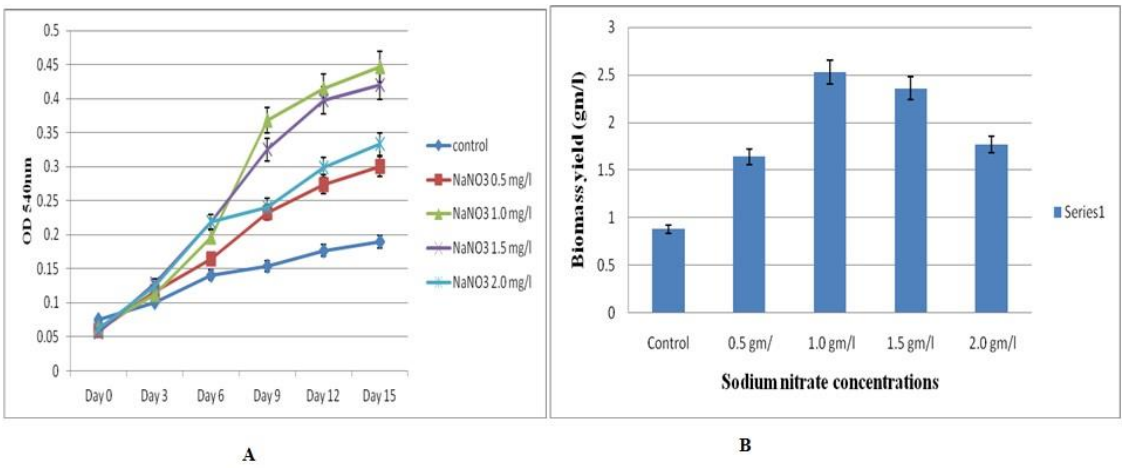


Fig 2: Effect of sodium nitrate on (A) Growth rate) (B) Biomass yield (dry weight)

Effect of sodium nitrate on lipid yield

Enhanced lipid productivity was obtained at 1.0gm/l concentration of sodium nitrate than other tested concentrations. Lipid yield of 0.585±0.14 gm/l and 0.403±0.17 gm/l was observed at 1.0gm/l and 1.5gm/l concentration of sodium nitrate respectively. At higher concentration i.e., 2.0gm/l of sodium nitrate, there was a significant decrease in lipid yield **figure 3**.

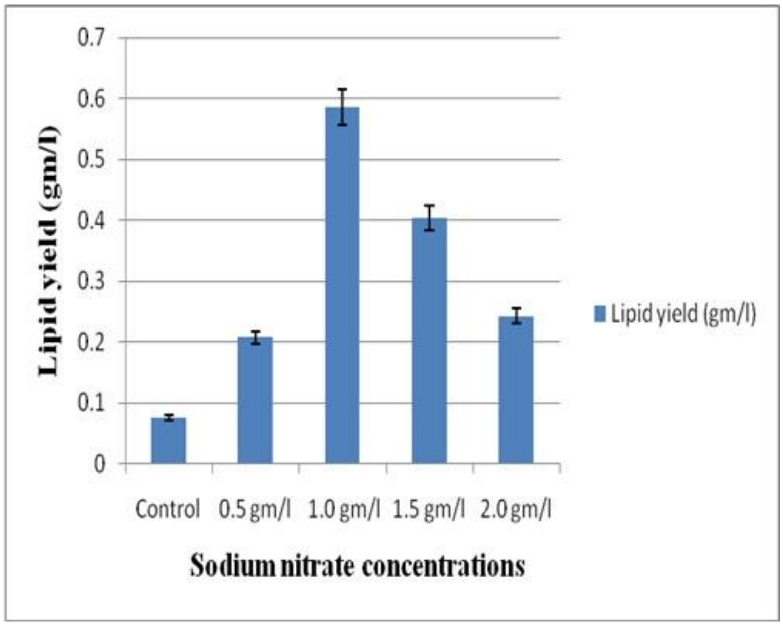
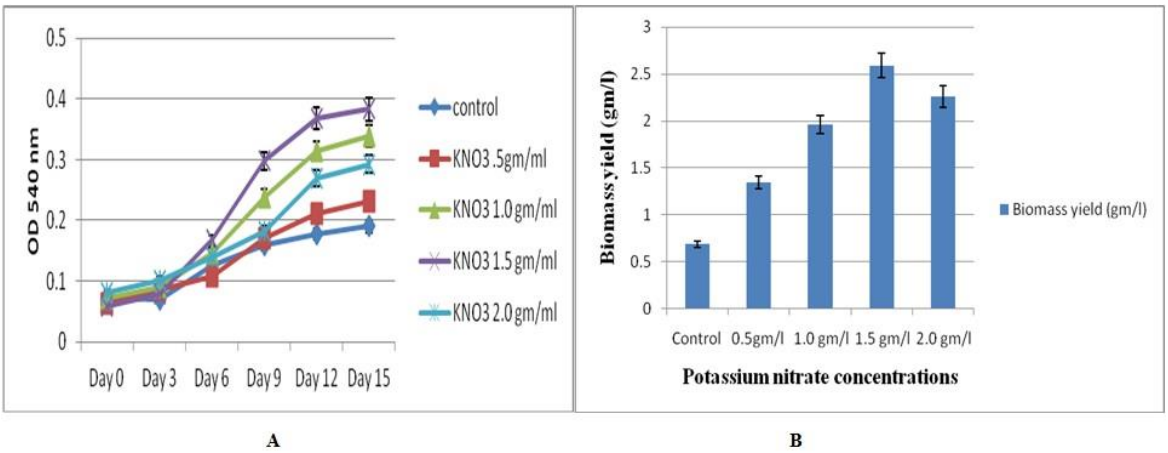


Fig 3: Effect of sodium nitrate on lipid yield

Effect of potassium nitrate on biomass growth

It was found that 1.5gm/l concentration of potassium was best to promote microalgal growth and biomass followed by 1.0gm/l and 2.0gm/l concentration, respectively. As 1.5gm/l of potassium nitrate was shown 2.59±0.04 gm/l biomass yield among all tested concentrations **figure 4.**



A B

Fig 4: Effect of potassium nitrate on (A) Growth rate (B) Biomass yield (dry weight).

Effect of potassium nitrate on lipid yield

At 1.5gm/l of potassium nitrate, acquired 0.524±0.10 mg/l lipid yield among all other tested concentrations. 0.437±0.47 gm/l of lipid was observed at 2.0 gm/l concentration of potassium nitrate, respectively **figure 5**.

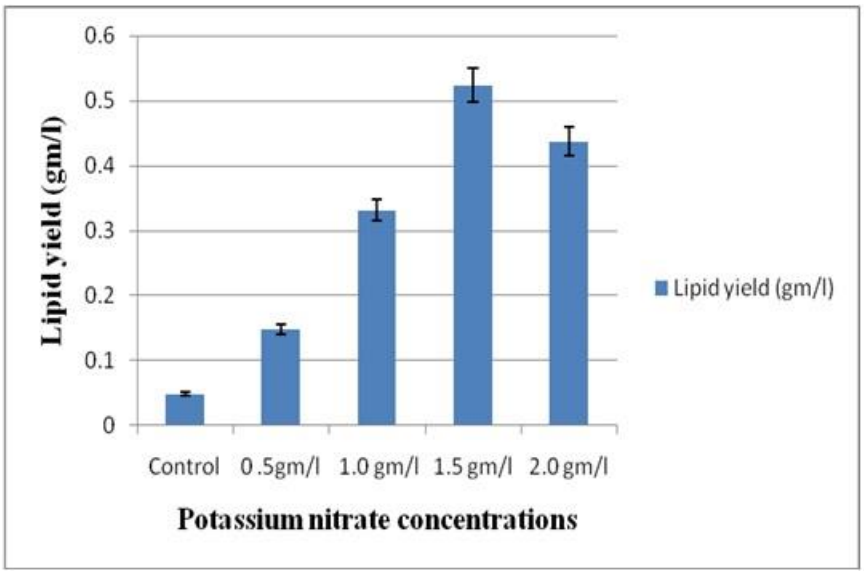


Fig 5: Effect of potassium nitrate on lipid yield

Effect of ammonium nitrate on biomass yield

All the four-tested concentration (including control) of ammonium nitrate performed relatively poor in growth and biomass yield. Higher biomass yield of 1.61±0.04 and 1.37±0.03 gm/l was obtained at 1.0gm/l and 1.5gm/l concentration of ammonium nitrate, respectively after 15 days of incubation **figure 6.**

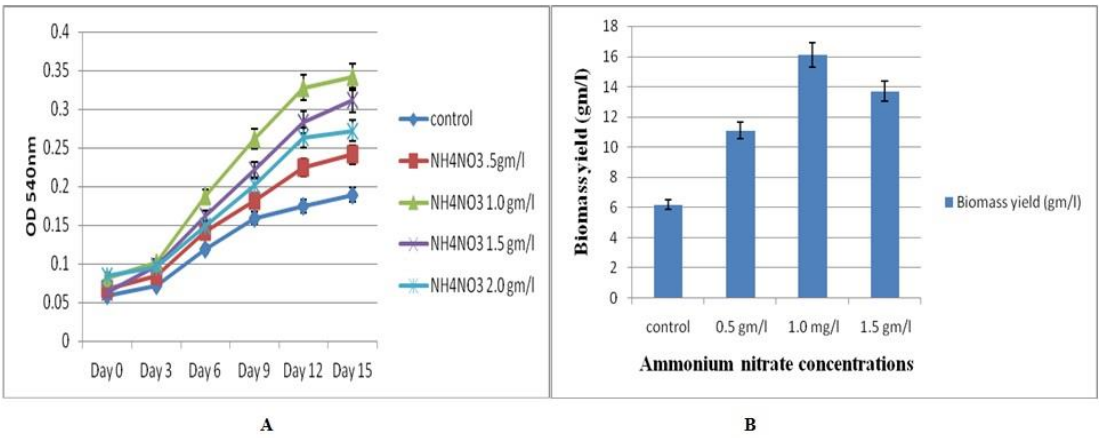


Fig 6: Effect of ammonium nitrate on (Growth rate), (B) Biomass yield

Effect of ammonium nitrate on lipid yield

Highest lipid yield of 0.152±0.10 gm/l was observed at 1.0gm/l of ammonium nitrate concentration followed by 0.122±0.61 gm/l and 0.106±.0.51 gm/l at 01.5gm/l and 2.0 gm/l concentration of ammonium nitrate, respectively **figure 7**.

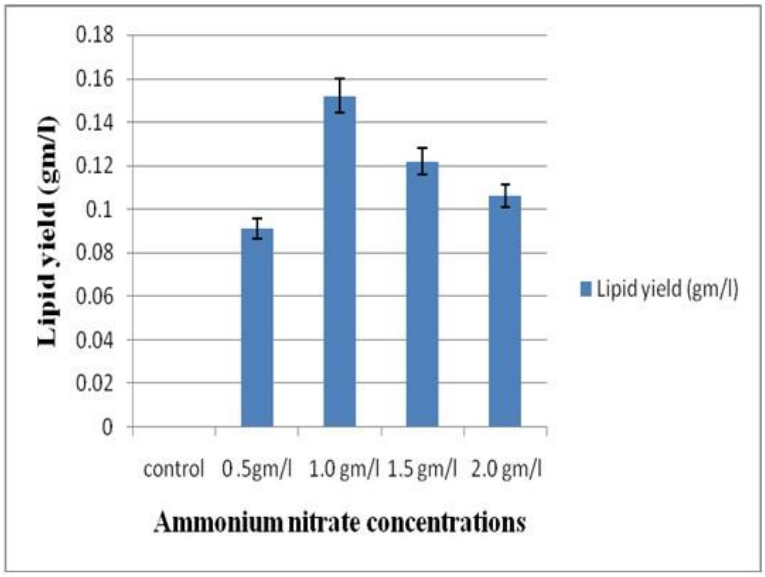


Fig 7: Effect of ammonium nitrate on lipid yield

Discussion:

The microalga *Scenedesmus sp*., investigated in present study, it was found to be suitable for sustained mass cultivation in laboratory conditions. Secondly, being an indigenous isolate, this organism would also have the flexibility to adapt in the environmental changes. When the *Scenedesmus sp*., was tested under laboratory conditions it grew luxuriously in BG11 medium and accumulated lipids at moderate levels. In recent years there are many reports related to growth and biomass content enhancement through different biotic and abiotic factors. Many abiotic factors like sulfur (Matthew *et al.,* 2008), nitrogen (Wang *et al.,* 2009; Work *et al.,* 2010), phosphorus (Weers *et al.,* 1997), zinc, or iron deficiency or other growth inhibiting stressors such as high salt (Siaut *et al.,* 2011) or high light (Zhekisheva *et al.,* 2002) were found to have a profound effect in causing a significant enhancement in the production

of biomass and lipid production (Griffiths and Harrison, 2009). It was found that nitrogen source potassium nitrate significantly enhanced the biomass yield at 1.5gm/l concentration (2.59±0.04gm/l) followed by sodium nitrate (2.53±0.03 gm/l) at same concentration. The findings are in accordance with the findings of Dayanand *et al.* (2006) and Li *et al.* (2008) where potassium nitrate was found to be preferred nitrogen source over other nitrogen sources for biomass growth of *Botryococcus braunii* and *Neochloris oleoabundans* respectively. Sodium nitrate and potassium nitrate and have shown almost similar trend of growth and performed better and ammonium nitrate exhibited moderate effect on growth and biomass productivity. The nitrogen sources responded well in order to enhance the growth but maximum growth was recorded for potassium nitrate indicating the favorable nitrogen source for the growth of green microalgae *Scenedesmus sp*. The possible reason for the better performance of potassium nitrate over other nitrogen sources may be due to the fact that it contains both nitrogen and potassium, two important nutrients for the algal growth. In contrast higher nitrogen (beyond 2.0gm/l) was not much responsive for microalgal growth as compared to lower concentration invariably in all the tested nitrogen sources. Last few years back, many studies has been reported to lipid/TAGs content enhancement through the depletion of nitrogen content in growth media. Maximum lipid was recorded 58.57±1.485 mg/l at 1.0gm/l sodium nitrate indicating the favorable nitrogen source for the lipid productivity of green microalgae *Scenedesmus* and in order to 52.48±1.070 gm/l at 1.5 gm/l potassium nitrate also showed almost similar affect on lipid productivity. *Scenedesmus sp*., showed a profound increase in lipid content (23.12 %) under sodium nitrate deprived conditions (1.5gm/l). Interestingly lower concentration of sodium nitrate showed better lipid productivity as compared to potassium nitrate as well as ammonium nitrate.

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