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Isolation and Screening of Microalgal Isolates for Biodiesel Production

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Production of Biofuels from algae is a promising technology which is alternative to conventional fossil fuels. Especially microalgae represent highly specialised group of micro organisms which are efficient in producing biomass and lipid through photosynthesis which can be used instead of petroleum, coal and natural gas. The present work aimed at the selection of an efficient microalgae for the production of renewable bio-fuels, sourcing out solvent for extraction, to investigate suitable parameters for production of lipids . Among the isolates of microalgae, *Chlorella* sp. MA-6 is promising in production of lipid and also in biodiesel yield as compared to *Chlorella* MA -1 and *Chlorella* MA-3. Under nitrogen limiting conditions, sodium nitrate at 1mM concentration could yield increased lipid content while it was decreased at low as well as high concentration. Among the solvents tested, hexane performed better than combination of Cyclohexane/Butanol in terms of yield in targeted glycerides. The isolate *Chlorella* MA-6 was suitable for production of biodiesel as renewable energy source.

Key words: Biodiesel, *Chlorella sp.* Microalgal isolates, fuel.

The Rapid depletion in the fossil fuels, coal, oil and natural gas and their impact on global warming by producing nitrogen, sulphur and carbon dioxide gases posed major challenge to mankind and necessitates the need for renewable energy sources for both ecological and economical sustainability (Rukminasari *et al.*,2013; Afity *et al*., 2010). For the past ten years, fuel production from algal biomass has received considerable attention from researchers and scientists as it is biodegradable, renewable and non -toxic fuel. Alternate energy resources like biodiesel are commercially produced currently from plants and animal oils, but not from photosynthetic organisms like micro algae (Yadavalli *et al*., 2012). Photosynthesis is the only processes that convert CO_2 into high energy rich organic compounds and provide a treasured source for sustainable fuel production (Sharma *et al*., 2012). Micro algae are sun light driven energy factories that are easy to cultivate and are efficient to convert CO_2, water to sugars and especially lipids to tryacyl glycerols (TAG's) in turn help to protect the environment from pollution (Afity *et al*.,2010). TAG's generally serve as energy storage in microalgae that, once extracted, can be easily converted into biodiesel through transesterification reactions (Fukuda *et al*., 2001).

Microalgae have been suggested as very efficient candidates for biofuel production because of their advantages of higher photosynthetic efficacy, higher biomass and faster growth compared to other energy crops (Miao *et al*., 2006; Widjaja 2009). They can provide several different types of renewable biofuels which include methane, biodiesel and bio hydrogen (Kapdan and Kargi, 2006). To enhance the commercial feasibility by using algal oil for biodiesel production, its very important to improve the algal biomass yield, lipid cell content and reach the downstream processing

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costs (Yadavalli *et al*.,2012). There are several ways to make biodiesel and the most common way is transesterification and the biodiesel produced can be used directly or blend with diesel fuel in diesel engine (Zhang *et al*., 2003).

In view of this, the present work is aimed to investigate, estimate the potentiality and sustainability of different microalgal species for biodiesel production using two different extraction solvent systems and also to estimate the effect of depletion in nitrogen concentrations on algal biomass and lipid production.

MATERIALS AND METHODS

Collection of samples

The collection of 59 water samples from different environmental locations were collected and subjected for the isolation of microalgal species.

Isolation of microalgae from water samples

A total of 59 water samples from selected lakes in north zone of Karnataka were collected and enriched in BG-11 medium for the growth of microalgal population in the water samples, and were incubated for 3 weeks in growth chamber maintained at 25000 lux light intensity , light and dark cycles (12:12) for 3 weeks. After conformation of the presence of algal cultures through the microscopic observations, the single cell culture was selected with a pastuer pipette and transferred to the fresh BG-11 medium and allowed to grow. Further purification was done by repeated streaking on agar medium. The purified cultures were maintained in liquid as well as on agar slants of BG-11 medium. The identification of the algal cultures was done by observing under the compound microscope upto genera level (Kumar , 1990).

Estimation of Biomass was done by gravimetric method (Richmond and Gobbelaaar, 1986) chlorophyll by solvent extraction method (Lichtenthaler and Buschmann, 2001) and for initial screening , extraction of lipid by chloroform/ methanol (2:1) was carried out for all 26 isoalates (lee *et al*., 1998).

Lipid extraction

Dry extraction procedure was used to extract the lipid in microalgal cells (Zhu *et al*.,2002). Dried microalgal powder (5g) was placed in a porous cellulose thimble which was placed in a Soxhlet extraction tube (Borosil) equipped with water cooled condenser and was suspended above a 500ml flask containing 250 ml solvent. The extraction procedure was allowed to proceed for 4h. Hexane (Boiling point 86^0C) was compared with combination of Cyclohexane/1-Butanol (2:1) as solvents. The solvent was evaporated and the mass of recovered extract was compared to initial dried algal biomass to determine percent recovery from extraction process.

Among all the isolates which are previously isolated, microalgal strains,*chlorella* strains MA-1,MA-3 and MA-6 were selected based on their biomass production and lipid yield and used for further experiment like lipid extraction, selection of extraction solvent, transesterification reaction.

Effect of nitrogen source

Nitrogen Limitation studies were undertaken on a large scale in 200 L capacity tanks to grow microalgae *Chlorella* MA-6 which showed maximum biomass and lipid yield. The Nitrogen source in BG-11 i.e Sodium nitrate was varied at four different concentrations as shown below:

Treatments	NaNO3 concentrations
T1	0.25mM $NaNO_3$
T2	1mM $NaNO_3$
T3	5.0mM $NaNO_3$
T4	10mM $NaNO_3$

The remaining nutrients in the BG-11 media were not varied

Transesterification of lipid from Microalgae

The lipid extracted using two different solvent combinations *viz*. Cyclohexane/1-Butanol and n-Hexane was transesterified using conventional two step method.

The lipid extracted after evaporating the solvent was charged with alkoxide solution of KOH alkali catalyst in a round bottom flask which was equipped with a condenser. The catalyst was used at the rate of 5% by weight of algae. Methanol was added at 12:1 ratio to the algal biomass. The mixture was heated in a controlled temperature of 60^0 C. The reaction was allowed to take place for 1 h and the flask was cooled to room temperature to stop the reaction.

The phase separation was carried out in Chloroform and water mixture at the ratio of 10:10:9. The mixture was shaken vigourously and

centrifuged at 2000 rpm for 10 min (Prommuak *et al.*,2012).

RESULTS AND DISCUSSION

The purified strains were identified based on morphological characters following the standard manuals. The identified strains are being presented in Fig 1. which belong to *Scendesmus sp., Botryochoccus sp., Chlorella sp., Diatoms , & Closterium* sp.

They were identified based on morphological characters following the standard mannuals. These microalgal isolates were then subjected to further analysis of biomass,lipid and chlorophyll content to screen the efficient isolate for further work.

The lipid content was estimated by

Table 1. Details of the isolates and their source of isolation

S. No.	Isolate No.	Source	Tentative identification of Microalgae
1	MA-1	Saligav lake	*Chlorella sp.*
2	MA-2	Sirsi lake	*Closterium sp.*
3	MA-3	Gokarna Sea	*Chlorella sp.*
4	MA-6	Kator lake	*Chlorella sp.*
5	MA-7	Tadur lake	*Diatom*
6	MA-8	Hanagal lake	*Chlorella sp.*
7	MA-9	Ranebennur lake	*Botryococus sp.*
8	MA-10	Sorab paddy field	*Scenedesmus sp.*
9	MA-12	Tadur lake	*Botryococcus sp.*
10	MA-15	Mundagod paddy field	*Scenedesmus sp.*
11	MA-17	Shingavi lake	*Diatom*
12	MA-19	Sorab lake	*Botryococcus sp.*
13	MA-20	Sorab lake	*Botryococcus sp.*

In the present study, 13 microalgal strains were isolated from 59 different water samples.

Table 2. Screening of the microalgal isolates for total biomass, chlorophyll and lipid production

Sl. No.	Microalgal isolate	Biomass ($mg\ L^{-1}$)	Total Chlorophyll ($\mu g\ ml^{-1}$)	Total lipid ($mg\ L^{-1}$)
1	*Chlorella sp.* MA-1	800	11.398	22.00
2	*Closterium sp.* MA-2	400	9.457	14.00
3	*Chlorella sp.* MA-3	800	14.658	23.00
4	*Chlorella sp.* MA-6	800	17.135	23.10
5	*Diatom* MA-7	725	16.131	20.03
6	*Chlorella sp.* MA-8	500	10.502	17.01
7	*Botryococus sp.* MA-9	200	9.511	10.14
8	*Scenedesmus sp.* MA-10	600	3.168	18.133
9	*Botryococcus sp.* MA-12	560	9.513	19.15
10	*Scenedesmus sp.* MA-15	521	10.361	18.00
11	*Diatom* MA-17	164	2.165	10.00
12	*Botryococcus sp.* MA-19	400	8.854	14.28
13	*Botryococcus sp* MA-20	428	5.018	17.00

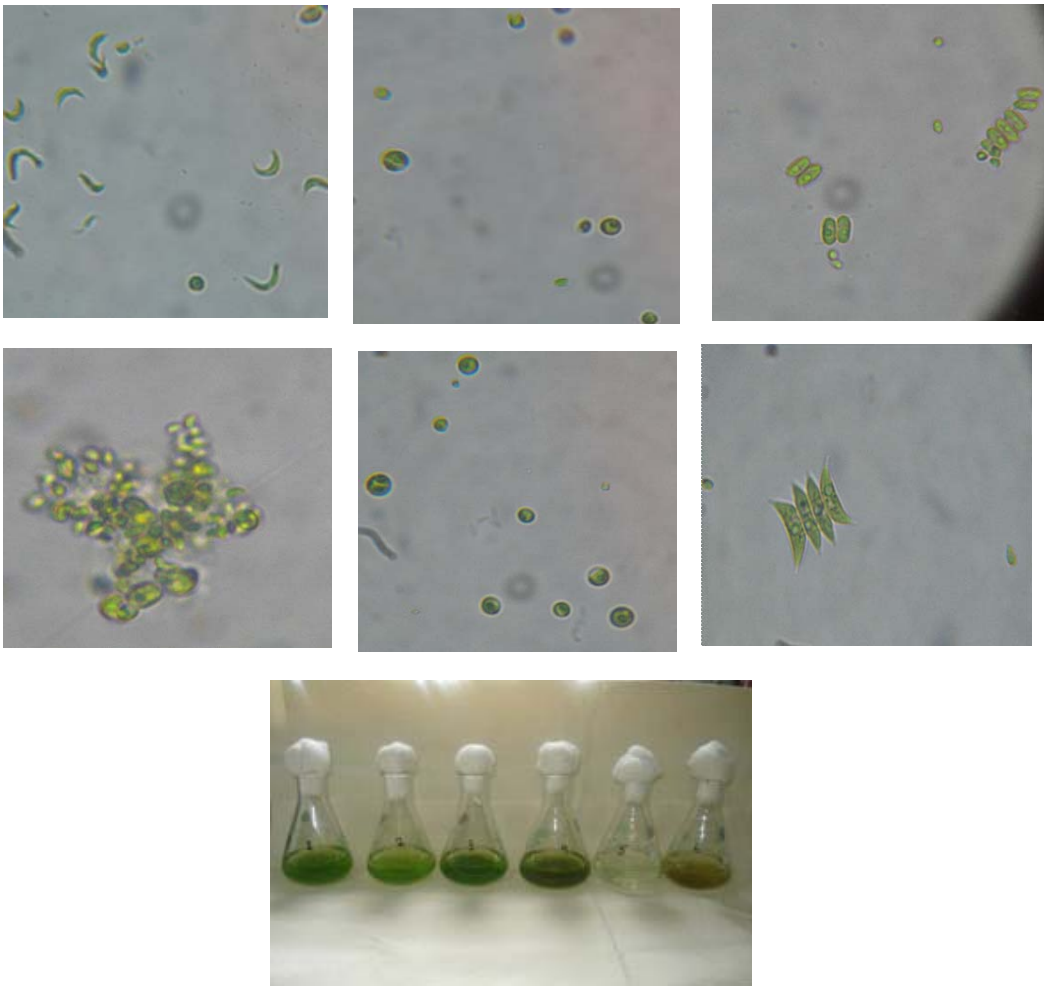
Maximum biomass being produced by *Chlorella spp*.MA-6, *Chlorella spp.MA-1and Chlorella spp.MA-3* that is,800mgL^{-1} and other isolates found to produce less biomass compared to *Chlorella spp*.

Table 3. Performance of isolates under open tank system.

Strain	*Chlorella* MA-1	*Chlorella* MA-3	Chlorella MA-6
Biomass production ($g\ L^{-1}$ dry wt)	0.56	0.63	1.1
Solvent	Hexane	Hexane	Hexane
Lipid %	7.6	1.4	9.0

Table 4. Comparison of solvents for extraction of lipids

Solvent	Ratio	Bioling point	Solvent volume	% Lipid Recovered
Cyclohexane/1-Butanol	9:1	80^0 C	250 ml	9.2
Hexane	-	60^0 C	250 ml	9.0



Gravimetric method and it was maximum in *Chlorella spp.*MA -6(23.10mg/l)and followed by *Chlorella sp.* MA-3 (23.00 mg L^{-1}),*Chlorella spp.* MA-1(22.00mg/l) and others found to produce less lipid. From studies it has been investigated that Chlorella species found to accumulate large quantities of lipids in cells under favourable conditions(Illman *et al*.,2000;Xiong *et al*.,2008;Xu *et al.*,2006).

Among all the 13 isolates,C*hlorella* strains MA-1,MA-3 and MA-6 were selected based on their biomass production and lipid yield and used for further experiment like lipid extraction, selection of extraction solvent, transesterification reaction.

From the table 3 it is evident that Chlorella MA-6 showed highest biomass production that is 1.1g L^{-1} dry weight followed by MA-3 and MA-1 which showed 0.63g L^{-1} and 0.56g L^{-1} of dry weight respectively. Hexane was used to extract lipid from micro algal strains and among these three micro algal strains Chlorella MA-6 showed high lipid content 9.0% followed by MA-1 and MA-3which had 7.6% and 1.4% respectively. From studies it has been investigated that Chlorella species found to accumulate large quantities of lipids in cells under favourable conditions (Illman *et al*.,2000;Xiong *et al*.,2008;Xu *et al.*,2006).

Extraction of lipid from *Chlorella* MA-6 which showed more biomass and lipid yield was carried out using two different set of solvents as shown in table 4. Among these, 250ml of mixture of Cyclohexane-butanol in the ratio 9:1 at 80^0C could extract 9.2% lipid compared to 9.0%using Hexane as solvent. Combination of Cyclohexane/1-butanol extracted highest amount of crude oil but hexane could be a better solvent in providing more desirable content of glycerides (Prammuak *et al*.,2012). In our study for the comparison of lipid yield of Chlorella MA-1,Chorella MA-3,and Chlorella MA-6 Hexane was used as solvent because the combination of Cyclohexane-Butanol was found to dissolve chlorophyll along with the lipid even though the yield of lipid content was slightly high. This indicates that hexane is a better solvent in terms of selectivity for targeted lipid. Therefore hexane was used in further investigations (Prommuak *et al.*,2012).

Table 5. Performance of MA-6 microalgae in various levels of sources of nitrogen s

Sl. No.	$NaNO_3$ concentrations	Biomass (g) (Dry wt.)	Lipid % (Dry wt.)
1	0.25mM $NaNO_3$	77.58	1.6
2	1mM $NaNO_3$	119.02	3.6
3	5.0mM $NaNO_3$	130.63	2.6
4	10mM $NaNO_3$	55.0	2.0

It is evident from the table 5 that algal biomass and lipid content of the cells varies with the concentration of nitrogen source. Biomass and lipid content of test algae was 77.58g and 1.6% respectively at 0.25mM concentration of sodium nitrate. Both were found to increase at 1mM concentration of sodium nitrate to 119.02g and 3.6% respectively. But at 5.0mM concentration there was increase in biomass to 130.63g and lipid content decreased to 2.6% of dry weight. High concentration of sodium nitrate resulted in rapid depletion in biomass as well as lipid content of the cell i.e 55.0g and 2.0% of dry weight. Thus, varying levels of nitrogen pose direct effect on algal biomass and lipid content (Afity *et al*., 2010).

According to Rajasri Yadavalli and her coworkers, the increased lipid cell content in lower concentration of sodium nitrate and decreased lipid content at higher concentration of sodium nitrate could be due to low initial nitrogen concentration in the medium will exhaust at low cell density since light can penetrate enough, resulting in enhanced metabolic flux from photosynthesis which might be channeled to lipid accumulation on a unit biomass basis. It suggests that cells accumulate large quantities of chlorophyll molecules when nitrogen source is abundantly available. But in the absence of external nitrogen sources, cells start to utilize chlorophyll as an intracellular nitrogen source.

Transesterification

Transesterification test resulted in the separation of two phases. Upper phase formed by methanol, by product of glycerol and other polar impurities dissolved in water while bottom phase contained fatty acid methyl esters, free fatty acids, lipid and other polar compounds dissolved in chloroform. Bottom phase was collected and stored as crude biodiesel.

CONCLUSION

From our study it is justified that the isolate *Chlorella* MA-6 is efficient producer of lipid and biodiesel from esterification of extracted lipid than compared to *Chlorella* MA-1 and *Chlorella* MA-3and can be used for production of biodiesel effectively. It is investigated that the isolate *Chlorella* MA-6 showed more lipid yield when the sodium nitrate concentration was increased slightly and it was less in presence of low concentration of sodium nitrate as well as in high concentration. This isolate showed highest lipid yield 3.6% at 1mM concentration of sodium nitrate. It is suggested that this could be the better concentration to get highest yield of lipid.

It is observed that hexane can be used effectively as solvent in lipid extraction rather than combination of cyclohexane/butanol because of its specificity towards the desirable contents of glycerides.

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