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PRODUCTION OF BIODIESEL FROM FRESHWATER ALGAE

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ABSTRACT

The production of biodiesel from micro algal lipids is studied by using newly isolated fresh water algal strains. Four different strains of *Chlorococcum*, *Deasonia*, *Chlorella* and *Botryococcus* species were isolated. The selection of the fastest growing specie was made through the results of growth kinetic studies when grown under optimum conditions. *Chlorella* sp. strain S-3 was grown in a large quantity of synthetic algal growth medium. This strain produced a large amount of oil (up to 22% of biomass), which was efficiently extracted by using (i) solvent extraction method through Soxhelet extractor with a 1:1 mixture of *n*-hexane and diethyl ether and (ii) by centrifugation of sonicated algal cells in liquid medium. By alkaline transesterification of algal lipids, biodiesel was formed as the main product with glycerol as by-product. The physicochemical quality of algal biodiesel was found comparable to conventional diesel.

Keywords: *Algae, Biofuel, Biodiesel, Transesterification, Kinetics, Chlorella*

INTRODUCTION

The use of fossil fuels causes the increase in global anthropogenic greenhouse gases (GHGs) (IPCC, 2012), which poses the detrimental impacts of climate change, such as sea level rise, drought, ocean acidification, and severe weather events (Arnell, *et al.,* 2013; Davis, *et al.,* 2013). To mitigate the rise in energy related GHG emissions, the replacement of conventional fossil fuels with bioenergy is considered the best strategy assuming that the consumption of biofuels does not cause any increase in net concentration of GHGs in the atmosphere over human time scales (Chisti, 2007).

Biodiesel is essentially the monoalkyl esters of long chain fatty acids, which is produced by transesterification of lipids obtained from biological materials (Lapuerta, *et al.,* 2008). The biodiesel obtained has similar physical and chemical properties compared to petroleum diesel, and hence can be used as an alternative fuel without doing any major modifications in the existing engines of the vehicles. It is an excellent renewable and safe alternative fuel with environment friendly nature as it contains low phosphorus and sulphur contents and produces 50% less carbon monoxide and 78% less carbon dioxide (Sheehan, *et al.,* 1998).

Among liquid biofuels, the optimization studies to produce ethanol (derived from fermenting glucose of sugarcane and corn) and bio-diesel (produced by transesterification of vegetable lipids of palm, soybean, rapeseed, and any other similar oils such as *Jatropha curcas*) have been widely conducted (Zeng *et al.,* 2008 and Kasim and Harvey, 2011). However, interests have grown in using microalgae largely due to containing high oil content (Chisti, 2007, Hu, *et. al.,* 2008 and Schenk, *et al.,* 2008).

Algae are a very large and diverse group of photosynthetic organisms, ranging from unicellular to multicellular forms. It can grow up to 65 meters in length (such as giant kelps). However, most of the algae are microscopic organisms. These organisms are photosynthetic and use energy from the sun, which is an important biochemical process in which algae converts the light energy to chemical energy using carbon dioxide and water to produce biomass (Meier, 1955 and Philip, 2011). Microalgae can be the source of a variety of renewable biofuels including methane, (Spolaore, *et al.*, 2006), biodiesel (Banerjee, *et al.,* 2002, Dunahay, *et al.,* 1996, Roessler, *et al.,* 1994, Sheehan, *et al.,* 1998), and biohydrogen (Kapdan and Kargi, 2006).

The idea of producing biofuels from algae is not new (Chisti, 1980–81, Nagle and Lemke, 1990). In the early 1950s algae was used to produce biogas by anaerobic digestion of the algal biomass (Meier, 1955). However, early researchers used wastewater as a source of nutrients for algae production. The concept of producing biofuel from algae not only gave a new life in scientific research to overcome the energy crisis, but it is now considered a reliable and sustainable way to accomplish the future demands of energy. More significantly, it is one of the most important and desirable components for mitigating

the anthropogenic greenhouse gaseous emissions in the ambient air (Gavrilescu and Chisti, 2005).

The biofuels, either obtained from plant seeds or animals has the disadvantage of higher raw material cost, low yielding and poor performances in varied atmospheric conditions. The polyunsaturated fatty acids of biofuel present a stability problem when used in the cold weather. Comparatively, algal biodiesel possesses better cold weather properties and is highly stable. Therefore, algal lipids may be considered as the highest yielding feed-stock for biodiesel production (Shay, 1993). In fact, microalgae can produce lipid over 200 times per acre of land as soya beans and up to 30 times higher than palm oil (Yusuf, 2007).

In present study, a *Chlorella* sp. was used to produce biodiesel. This strain was collected from the open storm water drain in the campus of University of Karachi, Karachi, Pakistan. In the first step, algal species were isolated from a mixed culture through enrichment culture technique. The oil from algae was extracted using a solvent system, while in the third stage, the oil was converted into biodiesel through transesterification reaction.

MATERIALS AND METHODS

The research on biodiesel production was jointly conducted in the Laboratories of the Department of Environmental Science, SMI University and Department of Applied Chemistry and Chemical Technology, University of Karachi.

Collection of water samples containing algal strains: A variety of micro algal strains were collected from fresh water. For this, water samples from open water ponds, storm water drains, water supply lines and water filtration plants were collected. A huge variety of algal strains were grown in the laboratory conditions.

Growth Medium and growth conditions: To isolate various algal species, agar-agar solidified medium was used. Growth kinetics of isolated strains were studied by using Bolds Basal (BB) Medium (Stein, 1973) that contained (g per litre): KNO_3 25, $CaCl_2.\ 2H_2O$ 2.5, $MgSO_4.\ 7H_2O$ 7.5, K_2HPO_4, 7.5, KH_2PO_4, 17.5, NaCl, 2.5, EDTA, 50.0, KOH, 31.4, H_3BO_3, 11.42, $ZnSO_4.\ 7H_2O$, 8.82, $FeSO_4.7H\ _2O$, 4.98, $MnCl_2.4H_2O$, 1.44, MoO_3, 0.71 1.57 $CuSO_4.5H_2O$, 1.57 and 1.0 ml H_2SO_4 (Analytical grade, Merck). The algal cells were grown under the following optimal conditions: temperature 20-28°C; salinity 24-28 g/l; light intensity 3000 lux; photoperiod (light: dark, 24:0 hr) and pH 8.5.

Isolation and identification of pure algal strains: Pure algal stains were isolated from mixed culture by enrichment culture technique. One millilitre of mixed algal culture was transferred to agar-agar plate (which was prepared by pouring molten agar-agar powder, 15 gm/l, Oxoid) and incubated for 5 days. Morphologically different colonies of algal cells were grown, which were separately transferred to 250 ml Erlenmeyer flasks containing 120 ml of BB medium and kept under light at room temperature with continuous supply of air through air blowing pumps. After 5 days of incubation a loopful of grown algal cells from the BB medium were streaked on agar-agar plates and incubated at room temperature under light. Pure colonies of algal cells were appeared after 5-7 days of incubation, which were further used to study the growth kinetics and production of biodiesel.

Pure algal strains were observed under light microscope at 1000X magnification. These cells were identified by comparing the morphology of individual cells of the isolated strains with the physical morphology of the standard algal strains listed in the standard method (APHA, 1992, p. 1141-1146).

Growth kinetics: Pure algal strains were separately incubated in 250 ml conical flasks containing 150 ml of BB medium under light at room temperature with continuous supply of air at non sterilized shaking conditions. To monitor the Optical densities (ODs) of culture medium, a uniform composition of culture medium was obtained by swirling the flask gently on a plane hard sur-face for several times. The ODs were monitored after every 24 h period at 680 nm with Shimadzu 1204 UV-Vis Spectrophotometer.

Monitoring of dry cell mass: Dry masses of the algal cells were monitored by filtering and drying algae. A known volume of culture was filtered on a nylon filter of 0.45micron pore size using a Büchner funnel connected to a vacuum pump. The salts were removed by washing the filter with a 0.5 M solution of ammonium formate. The same procedure was followed with control filters on which an equal volume of BB medium was filtered through the same filters. The filters were kept in the oven at 102°C for 1 h to evaporate ammonium formate. Finally, weight was monitored on electronic balance. The dry mass was calculated by using the following formula.

$$m = (m_a - m_c).\ (c.v)^{-1} \quad (1)$$

Where, m = dry mass (g. $cell^{-1}$), m_a = dry mass retained on working filter (gm), m_c = dry mass retained on control filter (gm), c = concentration of cells (cells.ml^{-1}), v = difference in volume of algal and control filtered media (ml).

Production of algal biomass and harvesting the pure specie: The isolated algal species were

grown in excess for the extraction of algal lipids. For this, 10 transparent plastic bottles were inoculated with the selected algal specie each containing 1.2 litres of BB medium and kept under optimal physical conditions.

After 3 weeks of incubation, algal cells were harvested by two different methods i.e. sedimentation and filtration. Sedimentation was done by adding commercial grade alum. Six Imhoff cones were filled with BB medium containing algal cells. Alum with the concentration of 20gm/L was added to each cone. The BB medium was stirred manually with a glass rod and left for 20 minutes. Algal cells were settled down and then separated by filtration using 0.45micron cellulose acetate filter paper through filtration assembly. In parallel, the clumps of algal cells were filtered through Whatman No.1 filter paper. The cells were scratched from the surface and dried at room temperature.

Cell disruption and extraction of algal lipids: Algal cells were disrupted by two methods i.e. (i) crushing and (ii) sonication. In crushing, the pellets of dried flakes of algal cells were first converted into powdered form by mortar and pestle and then the cells were broken by using a chopper, which was installed with a sharp knife for cutting the cells at 4000 rpm. Algal lipids were extracted by a mixture of 1:1 n-hexane and diethyl ether with Soxhelet extractor.

In second method, wet concentrated cells were disrupted by sonication using Labnics Equipment LU 5 Ultrasonic Bath (Operating Temp 40°C and frequency 40kHz, 1500W, 15min). Unbroken cells and cell debris were separated by centrifugation at 15,000 rpm using Beckman Coulter Allegra 64R Centrifuge. The supernatant of the centrifuge contained a mixture of lipids and water. Algal lipids were extracted by using Soxhelet extractor and separated from n-hexane at 70°C by using rotary evaporator (Buchi Inc.).

Production of biodiesel from algal lipids and its analysis: Algal biodiesel was prepared by transesterification of algal lipids. For this, a mixture of 0.25g NaOH (which acts as a catalyst) and 24 ml methanol was prepared in a 250 ml Erlenmeyer flask. This mixture was mixed with 100 ml of preheated algal lipids at 70°C. The reaction mixture was kept in an Erlenmeyer flask for 20 min under constant stirring using magnetic stirrer at a constant temperature of 70°C. The reaction mixture was cooled down to room temperature and kept in a separating funnel for 12 hours (Hossain *et al.*, 2008). The transesterification by product i.e. glycerol (lower layer) was separated from biodiesel (upper layer). To remove traces of glycerol from algal biodiesel, the product was washed with equal amount of water in a separating funnel. The washing was done many times until the product (biodiesel) became clear. The product was stored in a clean and moisture free container.

The physicochemical properties of biodiesel were analysed and compared with biodiesel standards published by the American Society for Testing Materials (ASTM) methods to verify whether the biodiesel fulfils the specification of standard diesel.

RESULTS AND DISCUSSION

Isolation and identification of algal strains: A variety of algal strains were isolated and identified in this study. A mixed culture of algal strains was growing at the surface of an artificial fresh water pond situated at the University of Karachi. The loopful of mixed algal cultures were collected in triplicates in sterilized glass bottles containing Bolds Basal (BB) Medium. The medium in the flasks were bubbled with atmospheric air through air pumps and kept under light for 24 hr at room temperature (20-28°C). The mixed algal cultures were then streaked onto the agar-agar plates and incubated under optimum conditions. A cluster of algal colonies was appeared in 7 days. The mixed culture was the combinations of several coexisting species including *Chlorella, Oscillatoria, Chloro-coccum, Deasonia, Ulothrix* and *Botryococcus*. Morphologically different colonies were separately incubated in the flasks containing BB medium supplied with air through the pump. Among various algal strains, four of them were selected to study the growth kinetics. These species were identified by observing their morphology under light micro- scope with the magnification of 10,000 X and designated as *Deasonia* sp. S-1, *Chlorococcum* sp. S-2, *Chlorella* sp. S-3, and *Botryo-coccus* sp. S-4 (Fig. 1), which were more abundant in the mixed culture than the other coexisting species. How-ever, various other algal species are used by some researchers to produce biodiesel. Hossain *et al.,* (2008) compared the production of algal oil and biodiesel in *Cladophora*, *Oedogonium* and *Spirogyra* species. It was reported that the algal oil and biodiesel production was higher in *Oedogonium*. However, biomass and sediments were found higher in Spirogyra than *Cladophora* and *Oedogonium* species (Hossain, *et al.,* 2008, Khola and Ghazala, 2012).

Growth kinetics: To select the fastest growing algae with the highest contents of lipids, growth kinetic studies were conducted. The isolated

strains were first grown on separate petri plates containing solidified agar-agar prepared in BB medium. A loopful of appeared algal colonies of *Deasonia*, *Chlorococcum*, *Chlorella* and *Botryococcus* species from the surface of agar-agar were inoculated into flasks S-1, S-2, S-3 and S-4, respectively containing BB medium. Increasing optical densities of the culture media were monitored at 680 nm.

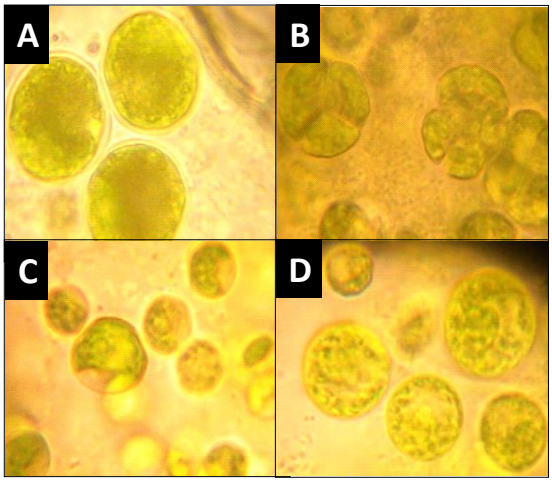

Fig. 1. Microscopic view of isolated algal strains through light microscope (magnification, X1000), (A) *Deasonia* sp. S-1, (B) *Chlorococcum* sp. S-2, (C) *Chlorella* sp. S-3 and (D) *Botryococcus* sp. S-4.

Figure 2A shows the growth kinetics of algal strains during five days of incubation under favourable conditions and figure 2B presents the flasks containing separately grown algal cells. It was observed that *Chlorella* sp., which was inoculated in flask S-3 was growing faster than the other species with the specific growth rate of 0.0224 h^{-1}.

Lipid content of Algal Species: In this study it was estimated that the *Chlorella* sp. S-3 contained 10-22% lipid content through the modified method of Ihsanullah, *et al.,* (2015). The maximum amount of oil content in any isolated *Chlorella* specie was not more than 55% (Chisti, 2007). However, *Chlorella protothecoides* contained 15-55 % of lipids (Wu, *et al.*, 2006) and another *Chlorella* specie contained 28-30 % of lipids (Sheehan, *et. al.*, 1998 and Chisti, 2007).

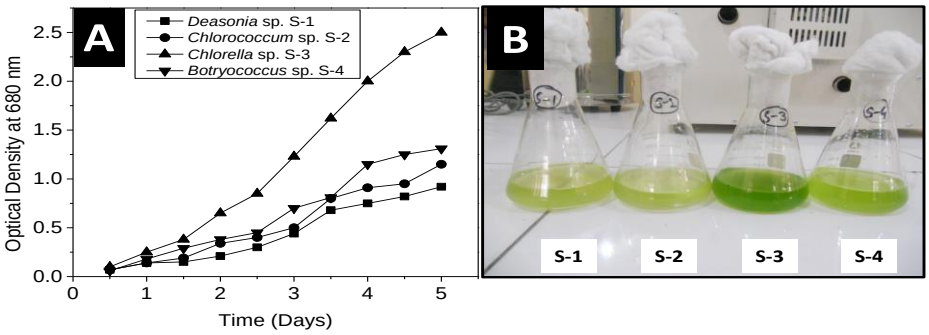

Fig. 2. Growth kinetics of four different algal species (A). *Chlorella* sp. S-3 grew faster than the other strains ((B), on fifth day of incubation), which was later grown in a large volume container. Algal lipids were extracted from the harvested cells and then biodiesel was produced by *trans*-esterification.

A heterotrophically grown *Chlorella protothecoides* accumulated up to 55% oil of its dry weight compared to only 14% in cells cultivated photoautotrophically (Miao and Wu, 2006), indicating that heterotophic conditions provide favourable growth conditions, which results in the formation of high cellular lipids content. Keeping in view of the fact that the *Chlorella* contained a reasonable amount of lipids and it grows faster than the other isolated species, we selected *Chlorella* sp. S-3 from among the isolated strains to grow in larger volume to extract lipids for the production of biodiesel.

Harvesting the algal cells: The selected algal strain (*Chlorella* sp. S-3) was grown in a relatively larger quantity of water (i.e., 80 litres) in a rectangular container under the optimum growth conditions. When the optical density of the medium was reached to the value of 10 at 680 nm, the algal medium was kept in Imhoff cones to harvest the algal biomass. Algal cells were settled down by adding alum in the medium. Each 1 litre Imhoff cone was added with up to 10g of alum, which is found to be the minimum amount for maximum settling of algal cells. The sediments were filtered in Whatmann filter paper (Fig. 3A). The pellets of algal biomass were then dried in an oven at 60°C. Alternatively, dried algal samples were obtained by centrifugation of cultures at 4,000 rpm for 20 min (Miao, *et al.*, 2004)

Extraction of algal lipids: To extract oil from algal biomass, two main methods were adapted. In first method, dried flakes of algal biomass were ground by using mortar and pestle (Fig. 3B), then the cells were broken by using a chopper installed with a sharp knife at 4000 rpm. The broken cells were treated with 100 ml of a mixture of 1:1 n-hexane and diethyl ether in a Soxhelet extractor

(Fig. 3C) under the reflux for 1 hour. The total lipid content was measured gravimetrically and reported as percentage on algae dry weight basis. The combination of two solvents yielded a higher fraction of oil extracted. The extracted oil was separated from the solvent in a rotary evaporator. The components of the recovered solvent was separated in two steps. amount for maximum settling of algal cells. The sediments were filtered in Whatmann filter paper (Fig. 3A). The pellets of algal biomass were then dried in an oven at 60°C. Alternatively, dried algal samples were obtained by centrifugation of cultures at 4,000 rpm for 20 min (Miao, *et al.,* 2004). Initially, the temperature was maintained at 35°C for 20 minutes to evaporate diethyl ether, then the temperature was raised to 70°C to extract n-hexane. Thus, the solvent free oil was left in the evaporation flask.

In parallel to this method, a second method was also adapted for lipid extraction. The algal cells were disrupted by a sonicator. The pulses of sonic waves caused the disruption of outer membranes of algal cells. Cell debris and unbroken cells were removed by centrifugation of cell lysate at X 8,000 rpm. However, Lee, *et al.,* (1998) developed a very effective method for the disruption of *Botryococcus braunii* UTEX 572 cells. They used a bead-beater to break the outer membranes of algal cells. The lipids were then extracted with a mixture of chloroform: methanol (2:1, v/v). This gave a lipid content of approximately 22% of dry weight. Soh and Zimmerman, (2011) used supercritical carbon dioxide ($scCO_2$) to extract components of interests from a fresh water unicellular green algae *Scenedesmus dimorphus* under a variety of algal harvesting and extraction conditions. They reported that the algal biomass harvested by centrifugation, displayed a similar extraction efficiency in $scCO_2$. These results suggested the potential energy conservation when the conventional algal mass dehydration was avoided prior to extraction. Furthermore, it indicates that $scCO_2$ is an environmentally friendly solvent, which shows great potential for sustainable algal oil extraction to produce biodiesel. However, Jones, *et al.,* (2012a) reported the comparison of various procedures for algal oil extraction. They found that 2-ethoxyethanol showed a higher oil recovery (up to 200%) compared to chloroform: methanol or hexane. Moreover, they studied the use of anion exchange resins to extract algal lipids (Jones, *et al.,* 2012b). Algal cells were bound and accumulated to anion exchange resins when the mineral salts medium contained with algal cells was passed through it, hence produced a dewatered concentrate. The algal oil was converted to biodiesel when the resin contained with algae was treated with the mixture of sulfuric acid and methanol and the resin was regenerated.

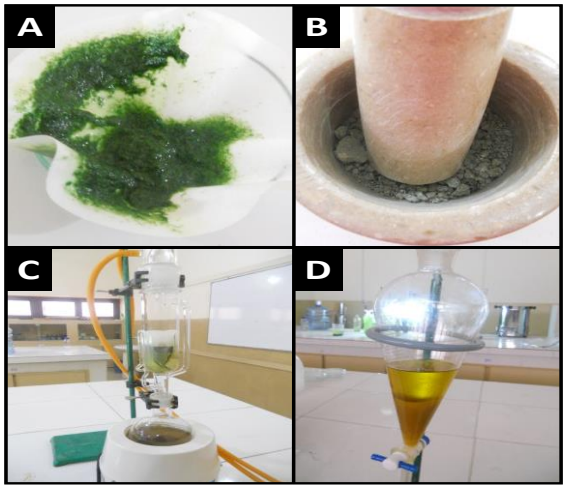

Fig. 3. Harvesting the algal biomass by sedimentation and then filtration through Whatmann filter (A). Dried flakes of algal biomass were ground with the help of mortar and pestle (B) before the extraction of algal oil through Soxhelet extractor (C). Two layer of biodiesel (upper) and glycerol (lower) were obtained through transesterification of algal oil (D).

Production and purification of biodiesel: The algal oil (contained triglycerides) was transformed into biodiesel through transesterification reaction in the presence of methanol (Fig. 4). To develop alkali-catalysed reaction, NaOH or KOH was used, which caused the completion of reaction in a relatively shorter period (Freedman, *et. al.,* 1984). In this process, triglycerides of the oil react with alcohol to form the fatty acid ester (biodiesel) and glycerol is formed as a by-product (Georgogianni, *et al.,* 2008). During this reaction, the algal oil reacted with methanol in the presence of alkali. Some researchers adapted a two-step alkali based pre-esterification and transesterifi- cation reaction if their oil contained high free fatty acid value (Awolu and Layokun, 2001 and Chen, *et al.,* 2012). Chen, *et al*, (2012) optimized the conditions for the chemical reaction to achieve 100% conversion rate of triglycerides to biodiesel. The molar ratio of 12:1 for methanol to algal oil caused to reach the highest level of biodiesel formation with 2% potassium hydroxide at 65°C for 30 min (Chen, *et al.,* 2012). Ahmad, *et al.,* (2013) used sodium methoxide as catalyst during transesterification and calculated 95% and 91% yields in *Chlorella vulgaris* and *Rhizoclonium hieroglyphicum,* respectively. However, under optimized conditions over 99% methyl ester content and about 95% biodiesel yield were obtained with a high oil content in *Chlorella pyrenoidosa* (Penglin, *et al.,* 2011).

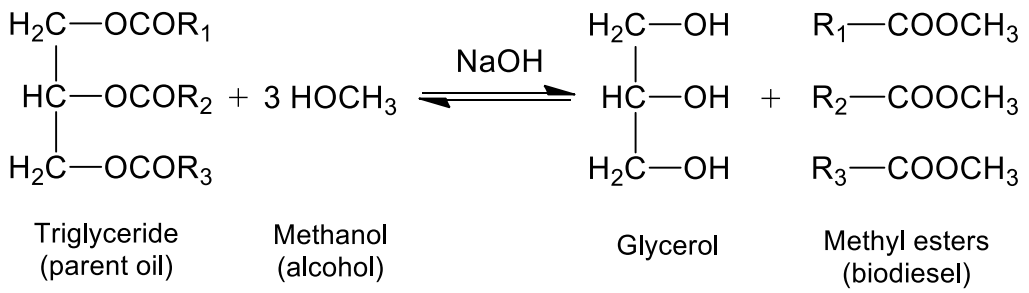

Fig. 4. Trans-esterification of triglyceride (algal oil) with methanol in the presence of alkali (NaOH).

The product (methyl esters) was separated from the by product (glycerol) by keeping the mixture in a separating funnel and let it stand for two hours (Fig. 3D). The lower layer (glycerol) was discarded and the upper layer (biodiesel) was kept in an Erlenmeyer flask. With gentle washing of the product using warm distilled water for remo -ving residual catalyst, methanol, salts, free glycerol and soaps, up to 99 % purified algal biodiesel was obtained. This helps in maintaining the product purity of 99.65%, to meet ASTM D-6751 of biodiesel specification. The product was kept in a clean container for further study.

Biodiesel analysis: The quality of biodiesel produced by transesterification of algal lipids was analysed by measuring its properties such as density, flash point, water content, kinematic viscosity, distillation and acid value (Table 1). The results of the selected parameters indicated that the quality of biodiesel obtained from the selected algal isolates was comparable with the prescribed ASTM standards. It was also observed that there was no significant difference in the properties of biodiesel produced in this study from *Chlorella* Sp. S-3 and the mixed algae culture reported earlier. Similar results were also obtained from the biodiesel of *Chlorella protothecoides* lipids to the conventional diesel fuel with ASTM 6751 (Li, *et al.*, 2007).

The higher flash point of biodiesel compared to petro diesel makes it a relatively safer fuel, hence it prevents the damages caused by catching fire in the vehicle during use or handling in the storage area. The viscosity of algal biodiesel was found higher than the petro diesel, however, it came under the limit of ASTM standard. The other properties of biodiesel are also very close to petro diesel. It suggests that the algal biodiesel can be used as a replacement of petro diesel, either as a fuel overall or blend with appropriate ratio with the petro diesel.

Table 1. Comparison of the physical properties of algal biodiesel with the standards and different types of diesels.

Properties		ASTM Test Method	Petro Diesel (Lapuerta, *et al.*, 2008)	Biodiesel (Miao and Wu, 2006)	Biodiesel (Ihsanullah, *et al.*, 2015)	Biodiesel (This Study)	ASTM Biodiesel Standard (Lapuerta, *et al.*, 2008)
Density (kg/l at 15°C)		D-4052	0.861	0.864	0.876	0.880	0.86-0.90
Distillation (°C)	50%	D-86	-		-	190	-
	90%		-		-	272	Min 340
Color		D-1500	-		-	L 1.5	-
Flash Point		D-92	60-80	115	140	160	Min 100
Kinematic Viscosity (mm^2/sec, cSt at 40°C)		D-445	1.3-4.1	5.2	4.9	5.6	4.0-6.0
Acid Value (mg KOH/g)		D-664	-	0.374	-	0.41	Max 0.5
Water content (ppm)		D-95	-		-	640	Max 500
Ash Content (% w/w)		D-482	-		-	0.002	Max 0.01

CONCLUSION

To develop sustainable and cost-effective methods to produce biodiesel from microalgae a successful study was conducted by using *Chlorella* sp. as a raw material. The process involved isolation and characterization of high lipids containing and fast growing algal specie, oil extraction from the selected algal strain and transesterification. It was observed that the maximum amount of oil was extracted from selected algal biomass using a combined solvent system containing n-hexane and diethyl ether. Various researchers have examined the production of biodiesel from different sources (edible and nonedi-

ble), virgin oil versus waste oil (Vasudevan and Briggs, 2008). Among all, algae-based biodiesel has gained increased importance specifically with respect to the role of different catalysts including enzyme catalysts. Biodiesel is found to be a better fuel than petro diesel. In comparison, biodiesel contains negligible amount of sulphur, higher flash poinnt, reduced aromatic content and it is bio degradable (Bala, 2006).

Improvement and optimization of algal oil production methods can be obtained by developing new culturing methods, effective methods for extracting oils from microalgae and optimizing algal oil composition for biodiesel production.

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