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Strain improvement of an endophytic fungal spp. for biodiesel production

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The *Aspergillus niger* JGK – 12 was subjected to mutation through physical and chemical methods (UV and EMS), and after treatment, the putative survived cultures were subjected to quantitative production of biomass and total lipid yield. Compared to control culture of *Aspergillus niger* JGK-12, UV treated mutant showed little enhancement in dry biomass content and total lipid production, whereas the EMS treated culture did not produce high biomass and lipid contents. The isolated lipids were then subjected to purification in which case, neutral lipids were found to be the major components accounting up to 85% of the total lipids, which infers that *Aspergillus niger* JGK – 12 could be a good microbial source for biodiesel production. The fractionated lipids were subjected to alkaline transesterification to produce biodiesel, which are mainly composed of fatty acid methyl esters and the compositions were found to be similar to that of regular biodiesel from plant origin. The biodiesel were further analyzed by GC-MS, HPTLC, ^{1}H NMR, C^{13} NMR and FTIR techniques to confirm their purity and quality. Hence, with all the research findings, it can be concluded that *Aspergillus niger* JGK – 12 can be treated as a promising feedstock for biodiesel production in the future.

Key words: Biodiesel, Mutation, Biomass, Lipids, Transesterification, Techniques

Strain improvement is an essential part of process development for microbial fermentation products. It is a means of reducing cost of developing strains with increased productivity (total yield), ability to use low value added raw materials, more specific advantageous characteristic such as improved filtration properties, ability to produce under particular conditions of temperature or aeration (Fakas*et al.,*2009). In spite of the development of newer techniques, such as rational screening and genetic engineering, traditional method of strain improvement by mutagenesis and selection on the basis of random screening still play important role as a reliable and cost effective method (Certik and Shimizu, 1999).

Although several wild-type oleaginous microorganisms are able to synthesize high oil, these strains have a limited ability to increase the formation of oil inclusions. Mutation techniques resulting in the suppression or activation of specific desaturases and elongases, which are beneficial not only for de-novo production of fatty acids, but they can also be useful for studying fatty acid biosynthetic pathway in oleaginous microorganisms (Ratledge and Wynn, 2002). There are various sources easily available which are natural oil containing

individual fatty acid precursors. Thus, mutants are excellent tools for regulating exogenous fatty acids flow to targeted lipids and unsaturated fatty acids. Moreover, mutants because of their simplicity of metabolism are considered to be excellent models for elucidating reaction mechanisms involved in fatty acid biosynthesis. Among oleaginous microbial strains, mutants of *M. alpina* with their unique fatty acid biotransforming enzyme system are probably the best studied (Ratledge, 2004). Among Zygomycetes, the Mucoralean moulds *Mortierella isabellina* and *Cunninghamella echinulata* show the ability to accumulate lipids when grown on glucose, pectin, starch or lactose as the carbon source (Papanikolaou *et al.*, 2007). Microbial lipids of *A. oryzae* contained major fatty acids such as palmitic acid (11.6%), palmitoleic acid (15.6%), stearic acid (19.3%), oleic acid (30.3%), linolenic acid (5.5%) and linoleic acid (6.5%) suggesting that the lipids be suitable for second generation biodiesel production (Muniraj*et al.*, 2013).

Many researchers have used alkali catalysts (NaOH, KOH, CH$_3$ONa) for production of biodiesel for their cheap and ready availability (Atapour *et al.*, 2011 and Demirbas, 2011). Arquiza *et al.*, 2000 have investigated biodiesel production from used coconut oil with methanol and NaOH as catalyst. In addition, transesterification of waste cooking oil with ethanol and NaOH as catalyst was evaluated by Chhetri *et al.*, (2008). Meng *et al.*, (2008) produced biodiesel from waste cooking oil with methanol and NaOH as catalyst. They investigated the effects of different operating parameters on conversion and quality of product. Besides, Jordanov *et al.*, (2007) investigated biodiesel production from waste cooking oil with sodium methoxide as catalyst and reported to have 85.5% yields of FAME.

Various analytical methods have been reported by the scientists for monitoring and compositional characterization of biodiesel, among these mostemphasized are chromatographic and spectroscopic methods of analysis.

A gas chromatograph coupled with a MS detection system is specially required for the diagnostic fragmentation of saturated and unsaturated FFAs and their corresponding esters, which would help in understanding their branching positions (Pinto *et al.*, 2005).These molecules elute from the GC column in the order of increasing their chain-length and degree of unsaturation. Sunflower oil biodiesel was characterized using Gel Permeation Chromatography (GPC) and GC by Madras *et al.*, (2004).

The advanced features of HPTLC facilitate the accurate analysis of biodiesel where HPTLC based analytical method plays a crucial role in the accurate determination of conversion percentage of oils into biodiesel and other byproducts (Chattopadhyay *et al.*, 2011).

NMR has been used to a) monitor the transesterification reaction used in the production of biodiesel, b) to monitor the oxidation of methyl esters in biodiesel and c) the ability of NMR to quantify blends of biodiesel and petroleum diesel (Diehl and Randel, 2007). Knothe and Kenar, (2004) have shown integrals of resonances in ^{1}H spectra can be used to determine the relative amounts of fatty acids in vegetable oils and methyl ester mixtures when the source of the oil feedstock is known.

FTIR is being employed as a modern analytical technique for detecting the conversion of biodiesel because it is a fast and easy detection method. Raw oils and methyl esters are noted as fairly strong absorbers in infrared region of electromagnetic spectrum. Ivanoiu *et al.*, (2011) performed a comparative study on biodiesel synthesis from different vegetable oils and used infrared spectroscopy using KBr plates in the range of 4000-400 cm^{-1}. Furthermore, Bergougnou *et al.*, (2009) produced biodiesel from *Jatroph acurcas* oil using potassium carbonate as the catalyst and

Bruker IFS 55 FTIR was used for analysis with an ATR (Attenuated Total Reflectance) cell made of ZnSe. They monitored the progress of the transesterification reaction by measuring the FTIR area (1446-1428 cm^{-1}) under the methyl (O-CH3) peak (1436 cm^{-1}).This area reflected the methyl esters of all types of fatty acids in the biodiesel.

The main criteria of biodiesel is to meet the international standards in terms of their physical properties. Density is an important property of biodiesel and it is also known as specific gravity. It is the weight of a unit volume of fluid. Specific gravity is the ratio of the density of a liquid to the density of water. Specific gravity of biodiesel ranges between 0.87 and 0.89. Fuel density directly affects fuel performance, as some of the engine properties, such as cetane number, heating value and viscosity are strongly connected to density. Contamination of the biodiesel significantly affects its density; therefore density can also be an indicator of contamination (Barabas and Todoruț, 2011). Viscosity affects the operation of the fuel injection equipment, particularly at low temperatures when the increase in viscosity affects the fluidity of the fuel. Biodiesel has a viscosity close to that of diesel fuels (Demirbas, 2006) esters.

The acid value (AV), also called neutralization number or acid number is the mass of potassium hydroxide in milligrams that is required to neutralize the acidic constituents in one gram of sample. The acid value determination is used to quantify the presence of acid moieties in a biodiesel sample (Berthiaume and Tremblay, 2006). Fuel that has oxidized after long-term storage will probably have a higher acid value (Barabas andTodorut, 2011).

The iodine value (IV) or iodine number was introduced in biodiesel quality standards for evaluating their stability to oxidation. Higher iodine value indicate an higher unsaturation of fats and oils (Knothe, 2002). The IV is a measurement of total unsaturation of fatty acids measured in g iodine/100 g of biodiesel sample, when formally adding iodine to the double bonds.

Materials and methods
Strain improvement for lipid production: 1. UV irradiation treatment 2. Ethyl methane sulphonate treatments were followed.

UV irradiation treatment: The UV irradiation treatment procedure was followed as per Elliaiah *et al.,* 2002. 72 hrs old fungal cultures were scraped off from agar slants and suspended in 5ml sterile distilled water and then diluted with 45 ml of sterile distilled water containing Tween 80 (1:4000). Sterile glass beads were added to the suspension and kept on rotary shaker for 30 min to break the hyphal mycelium. The suspension was filtered to remove the mycelium. The spore suspension was prepared in phosphate buffer (pH7.0) containing 106spores per ml. 5 ml quantities of the spore suspension were transferred aseptically into sterile petridishes and exposed to UV light (2600Å) at a distance of 15 cm away from the center of the Germicidal lamp for various time intervals from 0 min (control) to 5, 10, 15 and 20 min. The suspension was agitated by gently rotating the plates in between the time intervals. The UV exposed spore suspensions were stored overnight in dark to avoid photo reactivation. After overnight incubation, irradiated spore suspensions were serially diluted by using phosphate buffer (pH 7.0) and plated on potatodextrose agar medium. The plates were incubated for six days at ambient room temperature (28–30°C). The colonies were selected on the basis of their morphological characters and were given the code numbers ANuv0 (control), ANuv5, ANuv10, ANuv15 and ANuv20 (ANuv: *Aspergillus niger* JGK – 12culture treated with ultra violet radiation). Later, the putative UV mutants were inoculated to fat production medium (FPM) and incubated for 6 days at 30°C in incubator shaker(Industrial and

Laboratory Equipment & Co, Madras) to study the growth pattern.

Ethyl methane sulphonate (EMS) treatment: The EMS treatment was followed as per the procedure of Ellaiah *et al.,* 2002and Bapiraju *et al.,* 2004. Spore suspensions of fungal strains were prepared by using phosphate buffer, pH 7.0. To the spore suspension, individual aliquots of 1ml, a stock solution of EMS (50 µg/ml) was added, in different concentrations ranging from0 (control), 10, 20, 30, 40 and 50 µg/ml , then the cultures were vortexed gently and incubated for 5 min at ambient temperature (28 – 30°C). Later, samples were centrifuged at 2000 rpm for 10 min and the cells were washed three times with sterile distilled water and again re-suspended in 10 ml sterile phosphate buffer. The treated cell suspensions were serially diluted in the same buffer and plated on potato dextrose agar medium. The EMS treated mutants were given code numbers, ANems0 (control), ANems10, ANems20, ANems30, ANems40 and ANems50 (ANems: *Aspergillus niger* JGK – 12 culture treated with ethyl methane sulphonate). Later, the putative UV mutants were inoculated to FPM, incubated for 6 days at 30ºC in incubator shaker(Industrial and Laboratory Equipment & Co, Madras) to study the growth pattern.

Analytical methods
Estimation of cell dry weight: After fermentation, mycelium was harvested from culture broth by suction filtration through Whatman No. 1 filter paper. Cell dry weight was estimated by washing mycelia with distilled water twice and then dried at 55±2°C for 24hand fungal dry biomass was estimated.

Biomass harvesting and extraction of lipid: i) After 6 days of incubation period, the culture was filtered and washed with distilled water to remove any contaminants and was dried at 55° C for 24h, till it attained constant weight. The fungal dry biomass, total lipid yield and lipid contents were determined. The extractions of total lipids were performed according to Folch *et al.,* 1957.The dried fungal hyphae were subjected to lipid extraction using chloroform: methanol (volume ratio of 2:1) (Folch *et al.,* 1957). The fungal dry biomass, lipid yield were determined gravimetrically.

Fractionation of Single Cell Oils (SCO): Total neutral lipid content in the extracted SCOs of oleaginous fungal isolates were determined by lipid fractionation (Latge and De Bievre, 1980). Briefly, a known weight of total lipid extract was dissolved in 1mL chloroform and fractionated using a column (25 mm x 100 mm) of silicic acid (1g), activated by heating overnight at 110°C. Successive applications of 1,1,1 trichloro-ethane (100 mL), acetone (100 mL) and methanol (50 mL) were carried out for elution of neutral lipids, glycolipids plus sphingolipids and phospholipids, respectively, weight of each fraction was determined after evaporation of respective solvent fractions.

Production of biodiesel
Transesterification reaction was carried out by alkaline transesterification.

Alkaline transesterification: Preparation and analysis of FAMEs from fungal SCOs to analyze the fatty acid profile of the SCOs of oleaginous tropical mangrove fungi, the transesterification was carried out according to Leung *et al.,* (2010). The action was carried out in a 50 mL round bottom flask kept in a thermostatic bath with a reflux condenser and a magnetic stirrer using methanol to oil molar ratio of 60:1 and a catalyst (NaOH) concentration of 3 wt% relative to lipid sample. The reaction was carried out under ambient pressure for 90minutes at 60°C. The mixture was allowed to stand for 1 h to

collect the upper organic layer (FAMEs) and the solvent was removed by rotary evaporator (Industrial and Laboratory Equipments and Co., 70°C, 2hrs). The FAME samples were reconstituted in chloroform: methanol (2:1, v/v) and stored in clear screw top glass vials at -20°C till further use. To obtain the fatty acid profile of the transesterified fungal SCOs, all the samples were appropriately diluted with chloroform: methanol (2:1, v/v) mixture. The upper layer containing fatty acid methyl esters were taken and passed through an anhydrous sodium sulphate to remove the residual moisture content and solvent evaporated through nitrogen reflexing of the samples. After cooling to room temperature, FAMEs were extracted by hexane for two to three times and pooled samples reflected with nitrogen gas to remove any moisture content and stored in -20°C for further use.

Characterization of biodiesel
Gas Chromatography and Mass Spectrometry (GC-MS): Identification of fatty acid methyl esters were carried out using GC-MS (PerkinElmer 5906, Turbo mass Gold MS, Ausystem XL GC, Japan) at 70 eV (m/z 50–550; source at 230° C and quadruple at 150°C) in the EI mode with an BP-21capillary column (30 m, 0.25 mm i.d., 0.25 mm film thickness). Temperature program was set as follows: the initial temperature of the column was 120°C(for 1 min), then raised to 220°C at 5°C/min, and held for 10 min. Injection and detector temperatures were maintained at 240 and 250°C, respectively. Helium was used as carrier gas at a flow rate of 1 ml/min. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic standards and the spectral data obtained from the National Institute of Standards and Technology (NIST) libraries (Basumatary and Deka, 2012).

High Performance Thin Layer Chromatography analysis: The microbial biodiesel samples were subjected to HPTLC analysis (Gohel *et al.,* 2013). HPLC-grade methanol, n-hexane and ethyl acetate (EtOAc) were purchased from Merck SA (Riode Janeiro, Brazil) and used without further purification. The chromatography was performed on HPTLC silica gel 60 plates without fluorescent indicator (20x10 cm, Merck, Darmstadt, Germany). The plates were developed with a solvent system of n-hexane/ethyl acetate (92: 8v/v) in an automatic developing chamber (ADC 2, CAMAG, Muttenz, Switzerland) previously saturated with the same solvent system (20 ml) for 20min ambient temperature with total development length of 80 mm, the plates were then dried. Derivatization of HPTLC plates: The spots were derivatized by exposing HPTLC plates to fumes of Iodine crystals for 5 min. The plates were documented after derivatization, using a photo documentation system (Reprostar 3, CAMAG, Muttenz, Switzerland) under white light and short-wavelength light of 254 nm. All data were processed by Win Cats software 4.03 (CAMAG, Muttenz, Switzerland).

Fourier Transform Infrared analysis: FTIR spectra were obtained on a PerkinElmer (Norwalk CT) Spectrum 2000spectrometer equipped with a Galileo (Sturbridge MA) transmission-type fiber opticprobe. Quantitation methods were developed on a personal computer(Perkin Elmer, spectrum 2000). Method calibrations were carried out automatically by using the corresponding software feature (Knothe, 1999).

Nuclear Magnetic Resonance (NMR): Methyl ester quality was checked by NMR spectroscopy (Bruker ARX-400specrometer; Bruker, Rheinstetten, Germany) 400 MHz for 1H NMR and 100MHz for 13C NMR; solvent

CDCl3 to check for any possible contaminants in the sample.

Physical and chemical properties of biodiesel

Physical and chemical properties were determined by following the procedure of Indian standard methods of sampling and test for oils and fats, 1964 (Ved and Padam, 2013).

Acid number: The number of NaOH equivalents required to neutralize total free fatty acids present in 1.0 gm of the sample (Indian standard methods of sampling and test for oils and fats, 1964).

$$Acid\ number = 5.61\ x\ T/W$$

Where; T = Volume in ml of 0.5N NaOH required for titration, W = Weight in gm of sample taken.

Saponification number: A known quantity of oil is refluxed with an excess amount of alcoholic KOH. After saponification, the remaining KOH is estimated by titrating it against a standard acid.

$$Saponification\ number = 28.05\ x\ (T2 – T1)/W$$

Where; T2 = Volume in ml of 0.5N acid required for the blank, T1 = Volume in ml of 0.5 N acid required for the sample, W = Weight in g of the sample taken.

Iodine number: The fatty acid methyl ester is treated with an excess of solutions of iodine monobromide (IBr) and iodinemonochloride (ICl) in glacial acetic acid. Unreacted iodine monobromide (ormonochloride) is then allowed to react with potassium iodide, converting it to iodine, whose concentration can be determined by titration with sodium thiosulfate. The chemical reaction associated with this method of analysis involves formation of the diiodo alkane (R and R' symbolize alkyl or other organic groups).
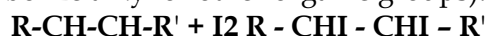
$$R-CH-CH-R' + I2\ R - CHI - CHI – R'$$

The precursor alkene (RCH=CHR') is colourless and so is the organoiodine product (RCHI-CHIR').

Iodine number =12.7 (B-S) N/W

Where; B = Volume in ml of standard sodium thiosulphate solution required for the blank, S = Volume in ml of standard sodium thiosulphate solution required for the sample, N = Normality of the standard sodium thiosulphate solution, W= Weight in g of the material taken for the test.

Kinematic viscosity: Kinematic viscosity (KV) of biodiesel sample was measured with the help of Redwood Viscometer No.1. Time of gravity flow of fixed value (50 ml) of sample was measured. The experiment was performed at 38 – 40 °C. Kinematic viscosity was calculated by the formula.

$$Vk =0.24\ t- 50/t\ for\ t > 100$$
$$Vk = 0.26t – 179/t\ for\ 34 < t > 100$$

Where; Vk = Kinematic viscosity (m^2/s), t = time of flow

Specific gravity: Specific gravity (SG) was measured using the standard method of Indian standard methods of sampling and test for oils and fats, 1964 Capillary stop per relative specific gravity bottle (pyknometer bottle) of 50 ml capacity was used to determine specific gravity of biodiesel at 30°C.

$$Specific\ gravity =A – B/B - C$$

Where; A= Weight in gm of the specific gravity bottle with sample at 30°C, B=Weight in gm of empty specific gravity bottle, C= Weight in gm of specific gravity bottle with distilled water at 30 °C.

Statistical analysis; Data obtained from three independent analyses was expressed as mean Standard deviation. Experimental data was subjected to analysis of variance and Duncan's multiple range test ($p < 0.05$) using the Statistical Analysis System (Duncan's, 1965).

Results and Discussion
Strain improvement for production of lipids in *Aspergillus niger*(JGK -12)
UV treatment combined with some chemical mutagens is mostly used to get hyper-producer strains of *A. niger*. Gamma-rays irradiation on *A. niger* mutants also improved the production of citric acid significantly (Majumder *et al.*, 2009).

UV irradiation treatment: Among the different time intervals of UV radiation exposure to the culture, the survival rates were decreasing as the time of exposure was increased, which might be due to the lethal effects of mutagen causing cell lysis. Compared to control, 5min exposed fungal culture comparatively gave more amount of cell dry biomass of 11.78±0.02 g/L (UV $_{5min}$) than control (non-treated) which produced 10.07±0.02g/L, and the total lipid yield also resulted in 3.87±0.23 mg than the control with 3.18±0.61 g/g, the lipid content was found to be 31.20±1.58 % and 29.01±2.48% respectively

(Table 1).The remaining plates with 10min, 15 min of UV exposure did not develop significant biomass in fat production medium and the plates with 20min exposure did not grow. Tembhurkar *et al.*, (2012) have reported that by inducing UV mutagenesis in *Aspergillus niger*, they are able to produce a mutant strain with high citric acid producing ability when the cultures were exposed to UV radiations to about 65 mins. Tapia *et al.*, (2005) have reported that upon UV irradiation treatment of *Lipomyces starkeyi*, the productivity increased by 15.1% in biomass and 30.7% in lipid productivity when compared to the wild-type strain with a similar fatty acid composition, despite a slight increase (approx. 7%) on the unsaturated fraction and concluded that the feasibility of the modified random mutagenesis strategies can be efficiently utilized for the genetic improvement of the oleaginous yeast *L. starkeyi*.

Table 1. Effect of UV radiation on the growth of *Aspergillus niger* JGK 12.

Sl. No	Time of exposure (min)	Dry biomass (g/L)	Total lipid yield (g/g)	Lipid content (%)
1	Control	10.07±0.02[d]	3.18±0.61[d]	29.01±2.48[d]
2	**5**	**11.78±0.02[e]**	**3.87±0.23[e]**	**31.20±1.58[e]**
3	10	2.31±0.02[c]	0.25±0.01 [c]	12.00±0.10 [c]
4	15	0.95±0.1[b]	0.04±0.01 [b]	5.43±0.92 [b]
5	20	0 [a]	0 [a]	0 [a]

Total lipid yield: *Total amount of lipid extracted in grams per total dry biomass of fungal mat in grams*
Values represent mean ± SD of three parallel experiments. In each column, mean values followed by the same letter are not significantly different according to DMRT at p < 0.05.

Ethyl methane sulphonate (EMS) treatment: The culture was treated with different concentrations of EMS ranging from 10 - 50 µg/mL for 5 min, the results indicated that, 40 µg/mL concentration of EMS was found to be optimum in giving 100% mortality rate and beyond, where the survival rates decreased with the increase in concentration of EMS. Among the different concentrations

of EMS treatment to the *Aspergillus niger* JGK - 12 culture, the survival rates were decreasing as the concentrations of EMS was increasing, and hence no growth was seen at 30 µg/mL and so on. The biomass of EMS treated cultures with 10 µg/mL resulted in 9.88 ± 0.06 g/L and total lipid yield of 2.9 ± 0.0 g/g, lipid content of 29.35± 0.19%with much lesser than the control culture

with10.07 ± 0.02 g/L of cell dry biomass and 3.09 ± 0.1 g/g, 30.70 ± 0.9% of total lipid yield (Table 2). Hence, due to the higher lethal effect of EMS, much biomass and lipid yield were not observed in treated cultures, whereas mutations introduced by Ethyl Methane Sulfonate enhanced the citric acid production to 3.2 fold in *A. niger* (Lotfy*et al.,* 2007).

The results were in concurrence with the findings of Gunashree, 2006, reported that EMS treatment of *Aspergillus niger* JGK -

12, did not support the growth of fungus. Since, EMS is an alkylating agent and highly mutagenic in lower organisms. The main effect of EMS on DNA is alkylation, in vitro and in vivo without activating cellular components. However, the main product, 7-alkylguanine is not necessarily the major cause of mutagenesis and the major mutagenic effects of EMS must be examined by biological means. The use of EMS as mutagen produces almost all kinds of genetic effects in all the organisms tested.

Table 2.Effect of EMS on the growth of *Aspergillus niger* JGK – 12

Sl. No.	EMS Conc. (µg/ml)	Dry biomass (g/L)	Total lipid yield (g/g)	Lipid content (%)
1	0 (Control)	10.07±0.02 [d]	3.09±0.1 [d]	30.70±0.9 [d]
2	**10**	**9.88±0.06** [c]	**2.9±0** [c]	**29.35±0.19** [c]
3	20	2.47±0.07 [b]	0.26±0.06 [b]	10.56±2.67 [b]
4	30	1.56±0.39 [a]	0.29±0.02 [a]	19.16±4.0 [a]
5	40	0 [a]	0 [a]	0 [a]
6	50	0 [a]	0 [a]	0 [a]

Total lipid yield: *Total amount of lipid extracted in grams per total dry biomass of fungal mat in grams Values represent mean ± SD of three parallel experiments. In each column, mean values followed by the same letter are not significantly different according to DMRT at p < 0.05.*

Purification and fractionation of lipids extracted from *Aspergillus niger*(JGK -12): The lipids extracted from the *Aspergillus niger*JGK – 12, was subjected to further purification to quantify neutral lipids, mainly triacylglycerol required for biodiesel production. The neutral lipid fraction varied from 65% to 85% (wt %) of 25.68 % to 19.78% of total lipids respectively. These values correlate with 85± 0.17 % (w/w) of the total lipids from 4 different extraction procedures indicating that the major components being neural lipids. The other fractions include Phospholipids constituting 10±0.40 % and glycol lipids with 1.2±0.23%.Oleaginous filamentous fungi *Cunnighamella ehinulata* contains 92% (w/w) of neutral lipid fractions as single cell oils (SCO) (Fakas *et al.,* 2007). Contrastingly, Vicente *et al.,* 2009 have reported that, in *Mucor circinelloids*, which

constitutes very low neutral lipid contents of about 18.5% (w/w) and 35% (w/w) of polar lipids(Table 3). In the present study, it confirms that the major fraction required for the biodiesel production is neutral lipids which constitute upto 90% of the total extracted lipids and the results are in agreement with the findings of Mahesh Khot *et al.,* 2012.

Table 3. Fractionation of lipids extracted from *Aspergillus niger* (JGK -12)

Types of lipids	Amount (%)
Neutral Lipids	85±0.17
Phospholipids	10±0.40
Glycolipids	1.2±0.23

Characterization of biodiesel
Gas chromatography and Mass spectrometry (GC-MS): The fatty acid methyl ester composition of Microbial biodiesel, synthesized by alkaline transesterification of the extracted lipids from dry biomass of Oleaginous fungal isolate *Aspergillus niger* JGK – 12, and its treated strains (UV and EMS). The GC- MS figure representing the Methyl palmitate (C-16) and methyl oleate (C 18:1) are shown in Figure 1 and 2 respectively. The fatty acid methyl ester content profiles are qualitatively similar but differ quantitatively among all the three strains (Table 4).

All the fungal fatty acid methyl esters in the present study were found to contain a high fraction of saturate and monounsaturated fatty acid methyl esters, importantly C- 16 and C- 18, which are considered as the potential features to signify the fuel quality of fungal biodiesel. This composition is in comparison with the biodiesel or fatty acid methyl esters produced from the commonly used vegetable oil feed stocks like rapeseed, soybean, sunflower and palm (Leung *et al.*, 2010). In contrast, the fatty acid methyl ester profiles of oleaginous algae and cyanobacteria show a dominance of C 14 and C 18 fatty acid methyl esters with Chlorella sp. being rich in C – 16 and C – 18 (Hu *et al.*, 2008). The fatty acid methyl ester profiles of biodiesel from the three strains namely, *Aspergillus niger* JGK – 12 (Control-untreated), *Aspergillus niger* JGK – 12 (UV$_{5min}$ treated), *Aspergillus niger* JGK – 12 (EMS 10µg/mL treated) were also found to differ from that of other oleaginous filamentous fungi. Among saturated fatty acid methyl esters, palmitic acid methyl ester (C 16:0) was found to be present in the highest quantity with values ranging between 18.6 ± 0.08 % for *Aspergillus niger* JGK – 12 control and 23 ± 1.28 % UV treated strain. Higher amounts of stearic acid were also found in the tranesterified oils of all the three strains with

the highest quantity of 23.4 ± 0.52% in control isolate and 21 ± 1.20 % in UV treated one. As per the previous reports, stearic acid contents have been reported from *Aspergillus* sp. ranging from 48 – 57 %, while very small amount were observed in *M. circinelloides* (7%) (Vicente *et al.*, 2010) and *M. isabellina* (1%) (Liu and Zhao,2007). Interestingly, low quantities of palmitic acids were detected in *Aspergillus* sp. (7%), while it was dominant in *M. circinelloid* (20.7 %) (Vicente *et al.*, 2010) and *M. isabellina* (28 %) (Liu and Zhao,2007).

Among the monounsaturated fatty acids, oleic acid (C 18:1) was found to be predominant in the microbial biodiesel among all the three strains, with highest content for EMS treated strain, 36.4 ± 0.70 and followed by remaining two strains with 34 ± 1.09 % in UV treated and 31.47 ± 0.49 % in Control untreated strain. Oleic acid contents have been found to be 0.1-1.6, 28 and 55.5% in lipids of *Aspergillus* sp, *M. circinelloide* and *M. isabellina*, respectively (Subhash and Mohan, 2011, Vicente *et al.*, 2010). Fungal lipids usually differ from most vegetable oils in being rich in PUFAs and hence are mainly exploited for PUFA production. However, PUFAs with more than 4 double bonds are not desirable for good quality biodiesel. In the present study, major PUFA member was found to be linolenic acid (C 18: 2) ranging from 16.4 ± 0.92 % in EMS treated strain and 22.1 ± 0.59 % in control strain. The quantity of linolenic acid (C 18:3) was found to be negligible and PUFA with four or more double bonds were not detected, are in agreement with the findings of Subhash and Mohan, 2011, in *Aspergillus* sp. (0.09%) and *M. isabellina* (2.4%). The other fractions were very negligible and hence contributing less to the properties of biodiesel which were varying from 2.6 ± 0.94 % in EMS treated strain and 3.4 ± 0.59 % in UV treated strain, where the control value was intermediate to the former ones.
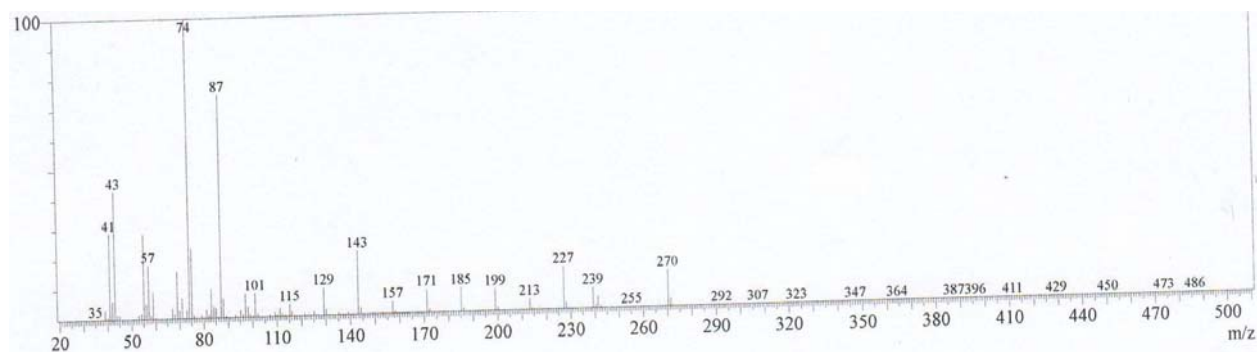
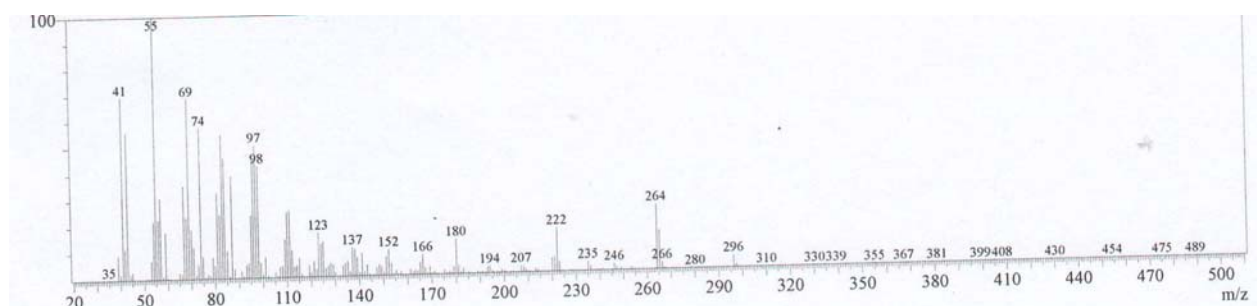Figure 1. GC- MS profile of biodiesel, Methyl palmitate (C 16: 0)

Figure 2. GC- MS profile of biodiesel, Methyl oleate (C 18: 1)

Table 4. Fatty acid methyl ester compositions of the microbial biodiesel

Sl. No	*Aspergillusniger*(JGK 12)	C:16:0	C:16:1	C:18:0	C:18:1	C:18:2	C:18:3	Others
1	Control	18.6 ± 0.08	1± 0.07	23.4 ± 0.52	31.47 ± 0.49	22.1 ± 0.59	0.4 ± 0.06	3.03 ± 0.39
2	UV treated	23 ± 1.28	0.4 ± 0.06	21 ± 1.20	34 ± 1.09	18.1 ± 0.51	0.1 ± 0.61	3.4 ± 0.59
3	EMS treated	22.2 ± 0.56	0.9 ± 0.12	21.2 ± 0.73	36.4 ± 0.70	16.4 ± 0.92	0.3 ± 0.08	2.6 ± 0.94

*Values represent mean ± SD of three independent trials. **C: 16:0**Palmitate, **C: 16:1**Palmitoleate, **C: 18:0: Stearate**, **C: 18:1**Oleate, **C: 18:2**Linoleate, **C: 18:3**Linolenate.*

High Performance Thin Layer Chromatography analysis: HPTLC was carried out by using CAMAG V instrument (Germany). Silica Gel G 254 plates were used as stationary phase, on which a known quantity of previously extracted fungal lipids and esterified products were applied. The plate was developed in hexane: diethyl ether (9:1). Keeping polarity in mind, non-polar solvent system was used for separation of non-polar compounds i.e. FAMEs, triglycerides, diglycerides and mono-glycerides. FAMEs being highly non-polar run fastest with Rf value 0.85 (track A and B) and were comparable in their Rf values of the biodiesel standards in track C, D and E (Figure 3). The present result confirms the finding of Gohel *et al.,* 2013, who reported that lipids could be easily separated on the basis of their chemical nature.
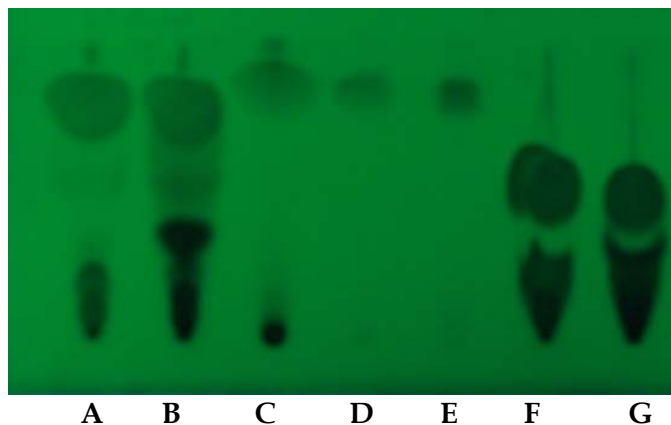
Figure 3. HPTLC of biodiesel. Developed in Hexane: Ethyl acetate (8:2) at 254nm. A and B: Microbial Biodiesel, C, D and E: Jatropha biodiesel, F and G: Microbial Oil

HPTLC plates: The UV detection of developed HPTLC plates of the separated biodiesel bands have shown that, the characteristic peaks of standard Jatropha biodiesel, at 290 nm (A) and 281 nm (B) are matching with the Microbial biodiesel sample (Figure 4A and B) thereby confirming FAME content of microbial biodiesel.
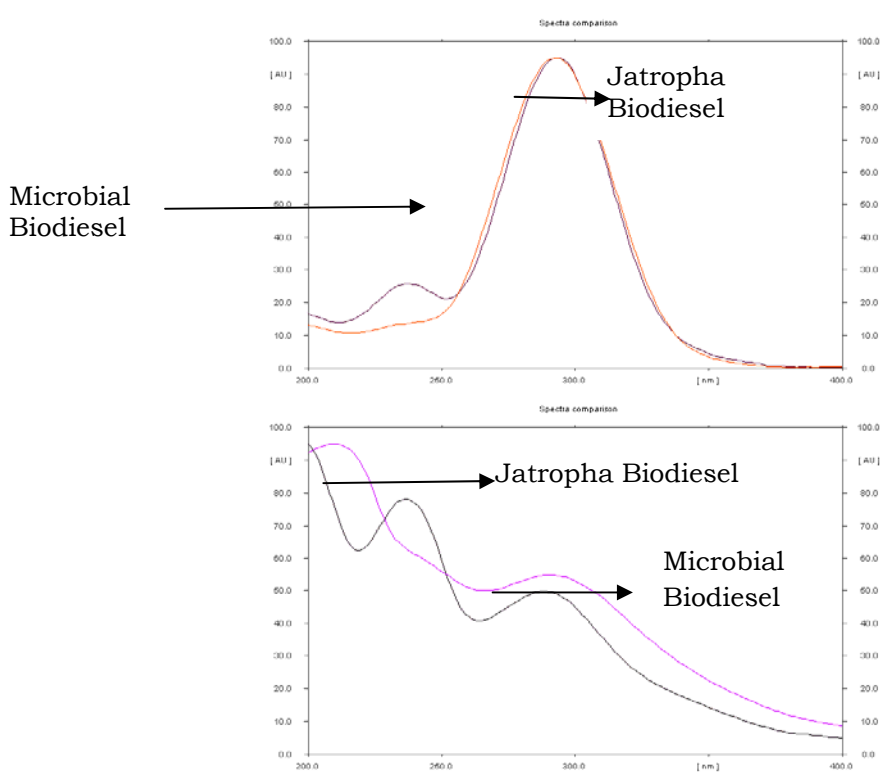

Figure 4(A and B). HPTLC spectra of biodiesel

Fourier Transform - Infrared analysis: The IR spectrum of biodiesel from *Aspergillus niger*JGK – 12, shown in Figure 5. IR spectrum of biodiesel showed a C=O stretching band of methyl esters at 1750 - 1735 cm^{-1} , which appears around the same frequency for methyl esters and triglycerides whereas stretching vibrations appear at

around 1700 cm^{-1} for long chain fatty acids (Albuquerque *et al.*, 2007 and Pavia *et al.*, 1996). Hence, the observation of this band in the spectrum of microbial biodiesel indicates the presence of the ester functional group. C - O stretching bands at 1171, 1197 and 1245 cm^{-1}. The weak signal at 1680 to 1620 cm^{-1} may due to C=C stretching frequency. Strong and sharp signals at 2850 and 2950 cm^{-1} are due to C-H stretching frequencies. The absorbance at 3015 cm^{-1} indicates the =C-H stretching frequency. The observation of an absorption peak at 735 cm^{-1} suggested the CH₂

rocking(Table 4.15), where the observations are in agreement with the findings of FT-IR data of biodiesel produced from lipids of marine micro algae (Patrícia *et al.*, 2013).

The FT-IR spectra in the mid-infrared region have been used to identify functional groups and the bands corresponding to various stretching and bending vibrations in the samples of oil and biodiesel (Mushtaq Ahmad *et al.*, 2011). The position of carbonyl group in FT-IR is sensitive to substituent effects and to the structure of the molecule (Safar *et al.*, 1994).
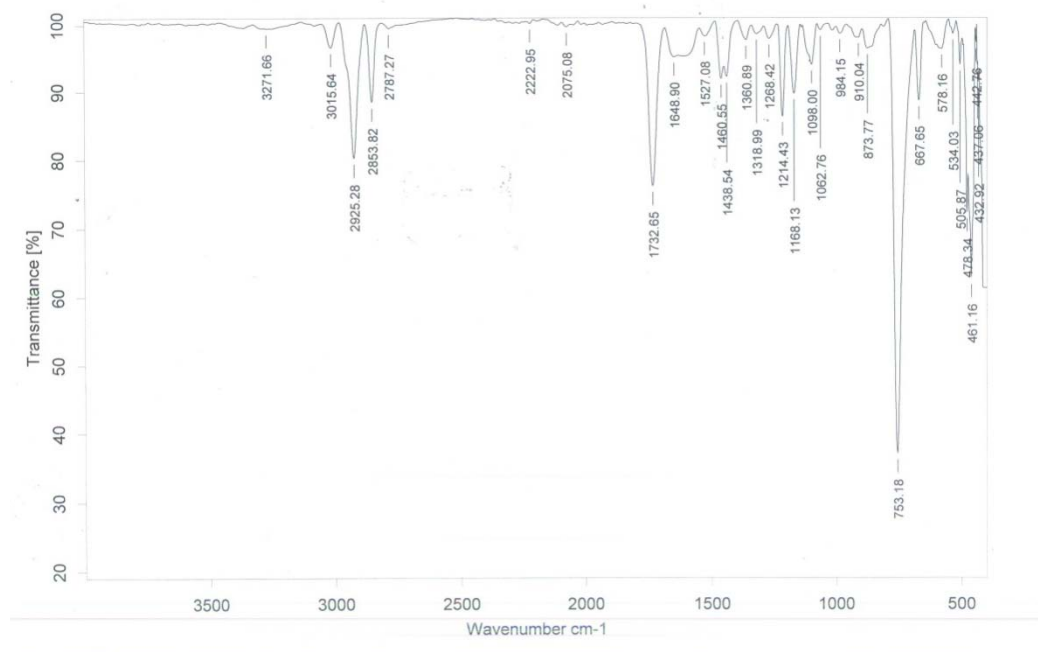

Figure 5. FTIR profile of microbial biodiesel

Table 5. Standard wave numbers for functional groups

Sl. No	Wave number cm⁻¹	Functional Group
1	1750 - 1735	Ester C = O Stretch
2	1680 - 1620	Alkenyl C = C Stretch
3	2950 - 2850	Alkyl C - H Stretch
4	3015 cm⁻¹	= C - H stretching

Nuclear Magnetic Resonance (NMR)
¹H NMR spectrum of biodiesel from microbial oil of *Aspergillus niger* JGK - 12
¹H NMR spectrum of biodiesel from Microbial oil of *Aspergillus niger* JGK - 12 is shown in Figure 6. The multiplet at δ 5.31-

5.36 ppm represents the olefinic protons (-CH=CH-). A singlet signal at δ 3.65 ppm is representing methoxy protons of the ester functionality of the biodiesel. The triplet at δ 2.76 ppm (t, 3J=5.7 Hz) indicates the bis-allylic protons (-C=C-CH2-C=C-) of the

unsaturated fatty acid chain. The triplet at δ 2.29 ppm (t, 3 J=7.5 Hz) represents the α-methylene protons to ester (-CH2-CO2Me). The α-methylene protons to double bond (-CH2-C=C-) appear as a multiplet at δ 1.99-2.05 ppm. The β-methylene protons to ester (CH2-C-COMe) also appear as a multiplet at δ 1.59 - 1.63ppm. The singlet signals at δ 1.24 and 1.30 ppm are expected for the protons of backbone methylenes of the long fatty acid chain. The terminal methyl protons (C-CH3) at δ 0.85-0.88 ppm appear as a multiplet. The bis-allylic proton signal of polyunsaturated fatty acid (like linoleic acid) generally appears around at δ2.8 ppm (Basumatary and Deka, 2012).

As per the findings of Swaminathan and Sarangan, 2012, the characteristic peaks of biodiesel were observed at chemical shift of 2.274 to 2.324 ppm in triplet with integration value of 0.96 that was characteristic of α-methylene protons adjacent to carbonyl carbon. A sharp peak of 1.42 was observed during chemical shift at 3.661 ppm which was characteristics of methoxy protons. Other chemical shifts occurred at 5.321, 5.338, 5.356, and 5.387 ppm in form of quaterlet which were characteristics of olefinic protons. A triplet at 2.7 ppm, characteristics of divinyl methylene protons, appeared with an integration value of 0.26. Moreover, a triplet occurred at 1.596, 1.618, and 1.640 ppm with an integration value of 0.92 which was characteristic of β-methylene protons from carbonyl carbon. Hence, all the present findings are well in accordance with the previous research studies.
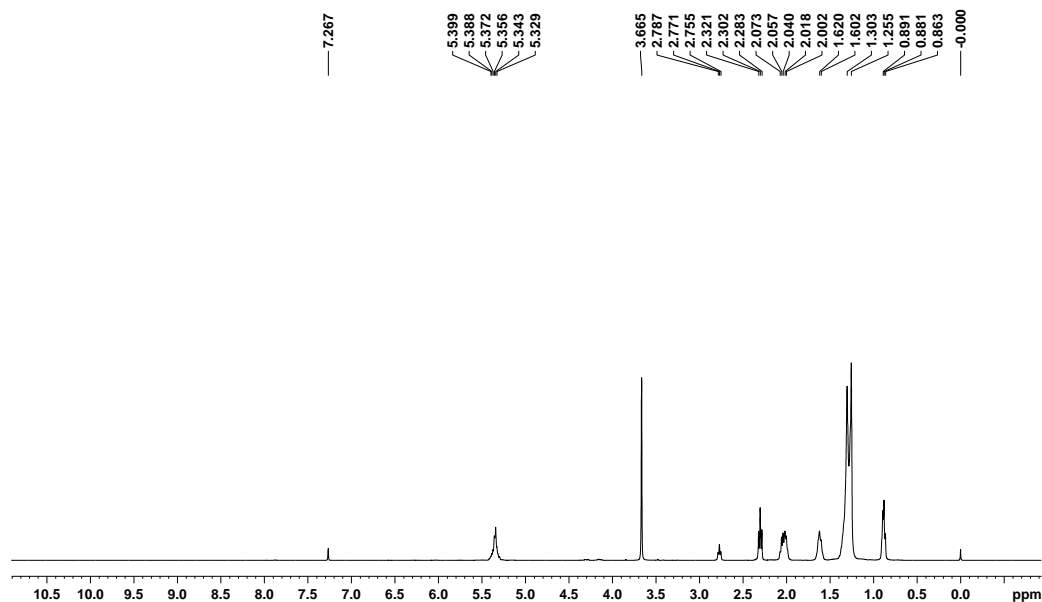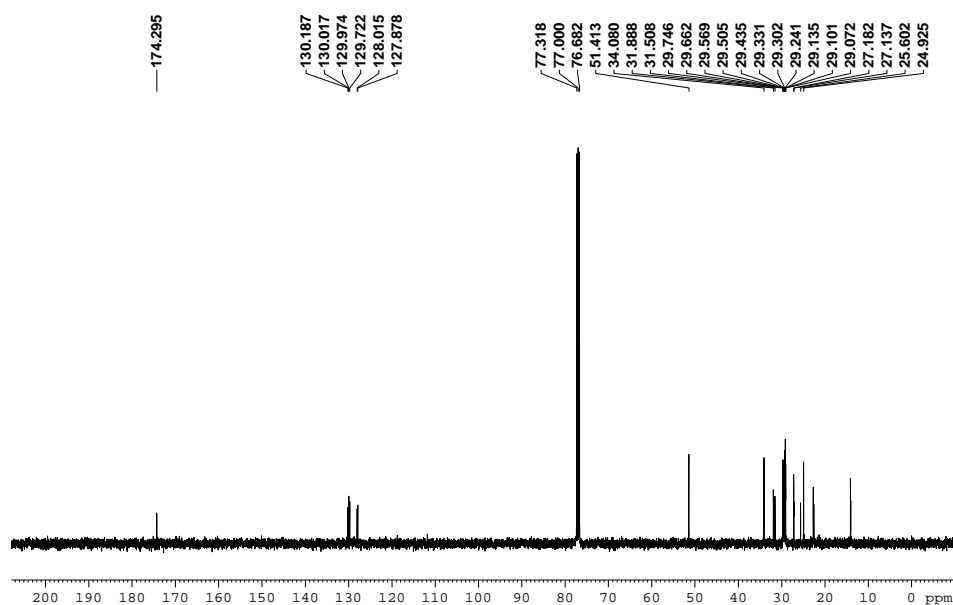

Figure 6. ^{1}H proton NMR profile of microbial biodiesel

^{13}CNMR spectrum of biodiesel from microbial oil of *Aspergillus niger* JGK - 12

The ^{13}C NMR spectrum of biodiesel from microbial oil of *Aspergillus niger* JGK - 12 is shown in Figure 7. The signal at δ 174.295 ppm represents the carbonyl carbon of the ester molecules of biodiesel and the olefinic carbons appear at δ 127.878, 128.015, 129.722, 129.974, 130.017 and 130.187 ppm. The signal at δ 51.413 ppm in the 13C NMR spectrum of biodiesel is due to the methoxy carbons of esters. The methylene and methyl carbons of fatty acid moiety appear in the range from δ 24.925 to 34.080 ppm. The results are corroborating with the findings of Basumatary and Deka, (2012) and Basumatary *et al.*, (2013).

Figure 7. C^{13} NMR profile of Microbial biodiesel

Physical and chemical properties of biodiesel: The acid number of microbial diesel has been found to be 0.12, which falls even less than Jatropha biodiesel, 0.24 and of regular diesel, 0.35. The saponification number was estimated to be 197, slightly higher than the Jatropha biodiesel, which might be due to the presence of long chain fatty acids in the TAG backbones. The experimentally determined iodine number of the microbial biodiesel was below the EN14214 specification (120 max) and iodine value of Jatropha biodiesel resulting in 98.5 and suggest good oxidative stability of the transesterified oils from *Aspergillus niger*JGK - 12. The viscosity of microbial biodiesel was estimated to be 4.36 mm²s⁻¹ which is lesser than Jatropha biodiesel (Padhi, 2010) and falls in the specified viscosity value ranges of ASTM D6751, EN 14214 and Indian biodiesel specifications (IS 15607). The density of the microbial biodiesel produced was found to be 0.815 g/mL, which is also permitted in accordance of the requirements of international biodiesel standards (Table 6). The calculated and experimentally determined SNs were found to be comparable for the microbial biodiesel of *Aspergillus niger* JGK - 12 while HHVs of ~ 40 MJ kg⁻¹ were similar to methyl esters of vegetable oils (Leung *et al.,* 2010) and the findings of the present study are very much in agreement with the finding of MaheshaKhot *et al.,* 2012. The biodiesel properties obtained in this study are well accorded with the findings of Patrícia Da Ros*et al.,* 2013, in assessing the conversion of cyanobacterial lipids for biodiesel production.

Conclusion

The present work enabled the identification of new fungal strain for its potential lipid production. An attempt is made to bring about strain improvement by UV mutation and EMS treatment, in which the mutants did not show much lipid production. The FAME analysis by sophisticated techniques like GC-MS, FTIR, HPTLC and NMR confirmed their suitability as biodiesel.

Table 6. Comparison of physical and chemical properties of biodiesel

Sl. No	Properties	Microbial biodiesel	*J. curcas* biodiesel	Diesel	ASTM D6751	EN 14214	IS 15607
1	Acid number (AN)	0.12	0.24	0.35	NS	NS	< 0.5
2	Saponification number (SN)	197	194	--	NS	NS	NS
3	Iodine number (IN)	98.5	101	--	NS	120max	NS
4	Kinematic viscosity (40^{0}C) mm^{2}s^{-1}	4.36	4.84	2.60	1.9-6.0	3.5 - 5.0	2.5-6.0
5	Density (gcm^{-3})	0.815	0.880	0.850	0.875	0.86 - 0.90	0.86-0.90
6	Concentrations of linolenic acid	0.7	--	--	NS	12max	NS

ASTM D 6751: *American biodiesel standard parameter,* **EN 14214:** *European Union biodiesel standard parameter* **IS 15607:** *Indian biodiesel standard parameter.*

This study succeeded in identifying an endophytic strain of *Aspergillus niger,* an oleaginous fungus that may find application in TAG production and provides useful basic information for further work on lipid production. The FAME content from the isolate has been proved to yield good quality biodiesel. From this point of view fungal lipids can be a supplement to other primary energy forms. The future course of this research would be to standardize bulk production of biodiesel from characterized oleaginous fungi.

Acknowledgement

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