Comparative analysis of biodiesel produced by acidic transesterification of lipid extracted from oleaginous yeast *Rhodosporidium toruloides*

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Received: 10 April 2018 / Accepted: 27 September 2018 / Published online: 3 October 2018
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Abstract

This study investigated the potential of oleaginous yeast *Rhodosporidium toruloides* strain (ATCC20409) for the sustainable production of microbial lipids as biodiesel feedstock and other economically important fatty acids in comparison to algal or plant-based biodiesel. The strain exhibited high lipid content (76% of dry cell weight biomass) through consolidated bio-processing which was transesterified to produce biodiesel. Physico-chemical properties of the biodiesel produced showed that they were in accordance with ASTM standards, although few parameters such as acid value, calorific value and free fatty acid value differed to some extent, as also reported in plant-based/microalgal biodiesel. Fatty acid methyl esters analysis of biodiesel showed 50.18% unsaturated fatty acid and 49.81% saturated fatty acid. Total content of (monounsaturated fatty acid) MUFA was higher than (polyunsaturated fatty acid) PUFA, being 44.36% and 2.69%, respectively. Considering the yield and cost, lipid extracted from *R. toruloides* may become a promising alternative feed in biodiesel production.

Keywords *R. toruloides* · FAMES · Waste extract · Biodiesel · Transesterification · Lipid · Fatty acid

Abbreviations

WE	Waste extract
HPLC	High-performance liquid chromatography
OD	Optimal density
DMSO	Dimethyl sulpho-oxide
FAMES	Fatty acid methyl esters
GC MS	Gas chromatography-mass spectrometer
FFA	Free fatty acid
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
FA's	Fatty acids

Introduction

The depletion of fossil fuel reserves, on the one hand, and a massive increase in energy demand, on the other, calls for a greater supply of fuels from renewable resources (Virginia et al. 2014). Due to change in climate, depletion of fossil fuels and energy security, petroleum alternatives for fuels used in transport sector are being sought to deal with changes in global warming and environment. As a result, the production of biofuels like bioethanol and biodiesel has increased worldwide for over 10 years. Biodiesel, which is derived from vegetable and animal fats, is mainly a mixture of fatty acid methyl esters (FAMES). Use of biodiesel has been increasing rapidly as an alternative source of energy (Meng et al. 2009). Transesterification of triacylglycerols using acidic or alcoholic method results in the production of unsaturated ethyl or methyl esters of fatty acids; this is known as biodiesel. The fatty acid composition has huge impact on the properties and consequently on the performance of biodiesel thereof (Knothe 2005, 2008; Steen et al. 2010).

Biodiesel has similar properties as petroleum diesel and might not require any modification in boiler engines apart from having few other lucrative properties such as non-toxic nature, renewability and sustainability. Apart from these, it

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-018-1467-9>) contains supplementary material, which is available to authorized users.

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delivers low sulfate emissions, and provides financial opportunities for provincial producers (Irnayuli et al. 2014). Few important properties of biodiesel often analyzed are its chain length, level of unsaturation, stretching change, cetane number, melting point, oxidative stability, kinematic consistency and heat of combustion.

The 1st generation biodiesel, usually produced from edible plant oils, was initially exhibited as fuel by Sir Rudolf Diesel. That type of biodiesel utilized oil or fats from peanuts (Crew 1963). Plant oils basically contained triacylglycerides (TAG) and unsaturated fats. These TAGs have been under investigation for more than 100 years (Nageli and Loew 1878). The increasing expense of edible plant oils and open verbal confrontation of the “food versus fuel” issue have supported improvement of alleged “2nd-age” biodiesel from unedible plant oils, for example, jatropha oil, jojoba oil and squander oils. On the other hand, the use of non-edible vegetable oils is of significance due to the obvious necessity of edible oil as food. The oils produced from these resources are unsuitable for human consumption due to the presence of toxic compounds in the oils (Banković-Ilić et al. 2012).

“Third-generation” biodiesel is generally obtained from oil-producing microorganisms, for example, microalgae, bacteria and yeasts. Single cell oils (SCO) are produced by oleaginous unicellular microorganisms by utilizing various waste materials as sources of carbon and nutrition (Sitepu et al. 2013). SCOs have been lately considered as subjects for active research, essentially in the light of increasing cost of oil.

Oleaginous microorganisms that can accumulate 25%–80% of their dry weight as lipids are considered as crude material for biodiesel production (Ratledge and Wynn 2002). Mainly, unsaturated fats: oleic, linoleic, linolenic and palmitoleic acids, are the significant unsaturated fatty acids commonly found in most yeast species, as analyzed from past investigations (Henderson et al. 2011; Kaneko et al. 1976; Sitepu et al. 2013; Viljoen et al. 1986; Woodbine 1959). In this study, we cultivated oleaginous yeast, which accumulated about 60% lipid of their dry biomass (Singh et al. 2016). These lipids are stored mostly in the form of triacylglyceride (TAG) in intra-cellular lipid bodies that may be detected by suitable staining (Singh et al. 2016; Chetana et al. 2015).

To achieve high performance, low-temperature operability and oxidative stability, biodiesel should contain low concentrations of both long-chain saturated FAMES and polyunsaturated FAMES (Verissimo and Teresa 2011). The two main features influencing the properties of biodiesel are (1) distribution of size and (2) the degree of unsaturation in the fatty acid structures (Hoekman et al. 2012). Therefore, the study emphasized on studying the properties of fatty acids extracted and on also on assessing the physical and chemical properties of the resultant biodiesel including pH, specific

gravity, water content, ash content, free fatty acid number, iodine value, refractive index and cetane number. We have also quantified the unsaturated and saturated fatty acids of the biodiesel produced.

Materials and methods

Chemicals and reagents

Reagents and chemicals used in this study, i.e. phenolphthalein, hydrochloric acid, methanol, toluene, chloroform, ethanol, iodine, thiosulphate, potassium iodide have been purchased from Thermo Fisher Scientific India Pvt Ltd. (Mumbai, India), HiMedia Laboratory Pvt. Ltd. (Mumbai, India) and Central Drug House Pvt. Ltd. (Mumbai, India).

Preparation of lipid and biodiesel

Preparation of growth medium: “waste” extract

Waste consisting of inedible parts of fruits/ and vegetables (e.g. peels of orange, sweet lemon, banana, mango, pea pods and stubs of cauliflower/cabbage), obtained from ‘mandis’, juice shops and various cafeterias, were pretreated for obtaining culture medium for *R. toruloides* (Chetana et al. 2015). The collected waste material was shredded; 30 g of it was steamed in 100 ml water for 15 min for preparing an ‘extract’ that would be utilized as fermentation medium (Suppl fig S1).

The various types of ‘mandi’ waste (orange/pea/ mango/ papaya peels; cauliflower/cabbage stubs) were analyzed individually for carbon (C) and nitrogen (N) content (data not shown). Peels with higher carbon content were added with those having appropriate N content to maintain the standardized C/N ratio of 30:1 in the resultant waste extract. C was determined by phenol sulphuric acid test (Dubois et al. 1956) and N by Kjeldahl method (Kjeldahl 1883). Methods related to waste extract (WE) preparation and pretreatment have been standardized and reported previously (Chetana et al. 2015).

Growth of *Rhodospiridium toruloides* strain ATCC20409 and lipid extraction

About 5 ml of 48-h-old seed culture (log phase) having optimal density (OD_{600}) in the range of 0.6–0.8 was used to inoculate 100 ml WE (Chetana et al. 2015) and incubated at 30 °C in an orbital shaker at 120 rpm. Samples were collected at every 6- to 12-h interval for determination of OD (600 nm), dry and wet cell weight, total sugar and reducing sugar of the medium as well as lipid content. Lipid was extracted using standardized protocol of Bligh and Dyer

(1959) during different growth stages like log, stationary as well as death phase and the lipid was analyzed as mentioned below.

Thin layer chromatography (TLC)

For TAGs analysis, the total lipid extract was dried under nitrogen (Speed-Vac ISS-100 vacuum evaporator) and resuspended in 100 μL of 6:1 (v/v) chloroform: methanol. This extract was applied to a silica 60 thin layer chromatography plate (Sigma-Aldrich) and neutral lipids were resolved using a solvent system of 70:30:1 (v/v/v) heptane: ethyl ether: acetic acid. Silica plate was stained with iodine vapors, in an adjacent lane that forms non-covalent brown complex bands with lipids. TAG bands were then identified by co-migration with a TAG standard extracted from soybean (Shaikh et al. 2017).

Transesterification of lipid for biodiesel production

For transesterification of the extracted lipid, 1 g of lipid was dissolved in 0.4 ml toluene, to which 3 ml methanol was added followed by 0.6 ml of methanol-HCl mixture. Mixture was vortexed gently for 5 min and incubated at 45 °C for 24 h. After incubation, upper layer containing biodiesel (yellow) was extracted by adding distilled water and toluene, until the lower layer looked clear and colorless.

Gas chromatography

Gas chromatography (GC) was done to quantify and identify individual components present in trans-esterified samples (FAMES). It was performed on a Rastech gas chromatograph model 2806 (Delhi, India) equipped with 30 m long GC column EC^{TM-1} (Grace Davison Discovery Sciences, Australia) containing dimethyl polysiloxane as the stationary phase. Lipid sample (2 μL) diluted in hexane was injected followed by a commercial standard FAME mix, CRM47885 (Sigma-Aldrich Co. LLC.) that was used for identification and quantification of FAMES.

GC-mass spectrographic analysis

Methyl esters of fatty acids were analyzed by a Gas Chromatography–Mass spectrometry (GC–MS) on a Shimadzu GCMS–QP 2010 Plus (Shimadzu, Japan) fitted with a SP–2560 capillary column (100 m $\times$ 0.25 mm i.d). The individual peaks were identified by comparison of their retention indices with standard chromatogram as well as by comparing their mass spectra with NIST/Wiley library of mass spectral database (Nandi et al. 2015).

Determination of physical properties

The physical properties like smell, color and physical state were assessed by sensory evaluation.

Determination of chemical properties

pH

The pH was determined using a pH probe (Broadley-James Fermprobe pH Electrode).

Ash content

The ash content was determined gravimetrically using a muffle furnace at 550 °C. The remaining ash content was determined to calculate the percent ash.

Free fatty acid and acid number

The free fatty acid (FFA) content and acid number were determined according to AOCS method (AOCS Official Method Ca 5a-40 1997). Oil sample (1 g) was weighed into a flask followed by addition of 95% ethanol and 2 ml phenolphthalein indicator. The ethanol was neutralized by adding NaOH until a faint pink color appeared. The sample was then titrated against sodium hydroxide until the appearance of a permanent pink color of the same intensity as that of neutralized ethanol (before the addition of the sample). As a precautionary measure, the permanent pink color was allowed to persist for at least 30 s during titration. The free fatty acid content and acid number was calculated as:

$$\text{Free fatty acid (\%)} = \text{ml of alkali} \times N \times 28.2/w,$$

where N is normality of NaOH solution, w is the weight of oil (g).

$$\text{Acid number (mg KOH/g)} = 1.99 \times \text{FFA (\%)}.$$

Iodine value

The Iodine value was determined using the AOAC official method (AOAC Official Method 993.20 2000). One flask containing 1 g of oil sample and second flask containing blank solution without oil as control were taken. In both flasks, 25 ml Wij solution and 25 ml chloroform were added and stored in a dry dark place for 1 h at room temperature. Thereafter, 20 ml KI solution and 150 ml distilled water were added to each. The solutions were then titrated against standard sodium thiosulphate until the yellow color of the solution became pale after which 1–2 ml of starch solution was added and the titration continued till the blue color of the solution disappeared. Iodine value was calculated using the following equation:

$$\text{Iodine value} = (B - S) \times N \times 12.7/w,$$

where B is the titer value of blank (ml), S is the titer value of sample (ml); N is the normality of hypo w is the weight of oil taken (g)

Refractive index

The refractive index was measured using Abbe's refractometer by placing 2–3 drops of the sample on the prism surface. The value obtained was recorded as refractive index.

Cetane number

Cetane number of a fuel is defined as the percentage by volume of normal cetane in a mixture of normal cetane and α -methyl naphthalene, which has the same ignition characteristics (ignition delay) as the test fuel (biofuel produced here), when combustion is carried out in a standard engine under specified operating conditions. It was calculated with earlier two values obtained from saponification value and iodine value. The cetane number was calculated using the following equation (Bose 2009):

$$\text{Cetane number} = 46.3 + 5458/\text{SN} - 0.225 \times \text{IV},$$

where SN is saponification number, IV is Iodine value.

Results

Lipid extraction and conversion of lipid to fatty acid methyl esters

Oleaginous yeast produces an enormous amount of lipid which can be further transesterified to produce biodiesel. Hence, after cultivating *R. toruloides* in WE, cells were harvested and the lipid was extracted using methanol: chloroform mixture via modified Bligh and Dyer method. Different batches were cultivated in WE with and without feeding the culture medium with glucose to estimate the conditions for achieving the highest cellular lipid content (Suppl. Table S1). The highest lipid content extracted from 38.75 g/l dry cell weight (dcw) of *R. toruloides* after growing in WE was determined as 76% (29.45 g/l), using optimized bioprocess. The lipid was then converted into biodiesel via acidic transesterification using sulfuric acid and methanol as catalyst and reactant, respectively (Fig. 1). Transesterification reaction converted the crude TAGs/FFA present in lipid to non-toxic, renewable and biodegradable biodiesel (Rawat et al. 2011). The extracted lipid (29.45 g) was transesterified to about 14 ml (10.5 g) biodiesel which is equivalent to 35.65% conversion rate (w/w). Similarly, oleaginous algal lipids have also been converted to biodiesel, although the amount of lipids extracted was comparatively

Fig. 1 Separation of biodiesel and glycerol layers during transesterification of lipid produced from *R. toruloides*

lower, owing to lower degrees of lipid accumulation or mechanical loss during the extraction process. Microalgal lipid content extracted from *Chlorella* sp. NT8a and *S. dimorphus* NT8e was reported as 14% and 8.2%, respectively, by Duong et al. (2015), which is quite lower than the reported value of 61.96% lipid obtained from another oleaginous yeast *Cryptococcus terricola* JCM 24523 when cultivated in soluble starch (Tanimura et al. 2014). Slininger et al. (2016) compared lipid production of microalgae *Chlorella* sp. strain NRRL YB-3399 to various oleaginous yeast strains and showed that *Chlorella* sp. produced only about 0.8 g/l lipid as compared to 8.8 ± 2.3 g/l produced by an oleaginous yeast strain *R. toruloides* Y-1091.

TLC (thin layer chromatography)

Thin layer chromatography (TLC) was performed on an adsorption plate (silica gel) which is principally applied for analysis of particular types of compounds such as polar lipid classes, including free fatty acid, triacylglyceride (TAGs), diacylglyceride (DAGs) and monoacylglyceride (MAGs) as described by Wang and Benning, (2011). Lipid extracted from different time intervals during the growth of *R. toruloides* were analyzed on TLC silica gel plate in triplicates. After exposing the TLC plate to iodine crystals, various spots were revealed indicating the presence of various fatty acids, TAGs, DAGs and MAGs, as shown in Fig. 2. The TAG standard (soybean oil) was a mixture of fatty acids, TAG, DAG and MAG which is crucial for production of biodiesel.

Fatty acid composition (GC-MS)

GC-MS analysis results revealed FAME profiles of oleaginous yeast *R. toruloides* (Fig. 3). The composition of various fatty acids present in the transesterified lipid/ biodiesel extracted from *R. toruloides* has been tabulated in Table 1. Saturated fatty acids comprised of 49.81% of total lipids including 9.96% tetradecanoic acid (C14:0 myristic acid); 21.1% hexadecanoic acid (C16:0 palmitic acid), 2.8% octadecanoic acid (C18:0 stearic acid), 3.39% of

Fig. 2 Thin layer chromatography analysis of lipid extracted from *R. toruloides* at different time points during its growth in waste extract

(C24:0) lignoceric acid and 0.33% of cerotic acid (C26:0). Unsaturated fatty acids were present in larger amounts as compared to saturated fatty acids. About 50.18% of total lipids were unsaturated FAs, mainly comprised of 17.59% oleic acid (C18:1, omega-9) MUFA with highest 11.07% of 9-octadecensäure, 12-hydroxy, methyl ester; total 13.54% of *cis*-10-heptadecenoic acid methyl ester (C17:1, heptadecenoic acid) MUFA and 2.18% of methyl 10-*trans*, 12-*cis*-octadecadienoate (C18:2, linoleic acid, omega-6) PUFA. Other valuable unsaturated fatty acids determined, consisted of 11.28% monounsaturated fatty acids, namely *cis*-10-nonadecenoic acid (Nonadecenoic acid C19:1). In the profile, other unsaturated fatty acids were also identified, namely 0.51% 1-eicosanol (arachidonic acid C20:4, Omega-6) PUFA; 0.22% 13-docosenoic acid, methyl ester (eucric acid C22:1, Omega-9) MUFA; 0.43% 3,5-Cyclo-6,8(14), 22-ergostatriene (nervonic acid C24:1) MUFA and 3.13% unusual cyclic aromatic compounds (Table 1). In other studies, the FAME components, methyl palmitate (C16:0), methyl oleate (C18:1), methyl linoleate (C18:2), and methyl linolenate (C18:3) were the main components observed in FAME analyses. The results are comparable to reported studies by Tang et al. (2011) with *Chlorella*

minutissima. Also, in another study, Wahlen et al. (2013) compared results from a microalgae (*Chaetoceros gracilis*), yeast (*Cryptococcus curvatus*) and a bacterium (*Rhodococcus opacus*) which showed that microalgae and oleaginous yeast serve as better sources for production of fatty acids used for biodiesel production, as compared to bacteria. Bowen (2010) suggested that the presence of saturated fatty acid is beneficial for biodiesel production but its high melting point can lead to gelling of fuel in cold weather. Unsaturated fatty acids, on the other hand, have low melting point which makes them most suitable for biodiesel production and helps them withstand in cold climates, but they are more prone to oxidation and rancidification unlike saturated fatty acids (Bowen 2010). In our study, FAME profiling results indicated high linolenic acid content (average of 22.95%), which would provide oxidative stability to the biodiesel, as low levels of linolenic acid otherwise may lead to poor stability of fuel (Chisti 2007). The presence of both palmitoleic fatty acids and myristic acid is beneficial as palmitoleic acid improves oxidative stability without sacrificing cold flow performance (Knothe 2008) and shorter chain fatty acids such as myristic acid improves NO_x emissions (Knothe 2005).

Fig. 3 GC-MS (Gas chromatography- Mass spectrometry) analysis showing different fatty acid peaks of biodiesel produced from *R. toruloides*

Table 1 Fatty acid composition (FAMES) as determined by GC–MS analysis of biodiesel produced from *R. toruloides* when cultivated in waste media

S.no.	Common Name	Retention time (Mins)	Area (wt %)
Saturated Fatty acid			
1.	Undecyclic Acid (C11:0)		
	Undecanoic acid, 10-methyl-, methyl ester	8.977	0.57
2.	Myristic Acid (C14:0)		
	1-Tetradecanol	13.196	1.64
	Tetradecanoic acid, methyl ester	14.560	9.96
	1-Trimethylsilyloxytetradecane	15.462	1.33
	Tetradecanoic acid, trimethylsilyl ester	17.666	1.27
	Myristyl myristate	40.608	0.43
3.	Palmitic acid (C16:0)		
	Hexadecanoic acid, 3-hydroxy-, methyl ester	18.387	0.79
	Hexadecanoic acid, methyl ester	19.944	21.11
	Hexadecanoic acid, 15-methyl-, methyl ester	22.071	0.24
	Hexadecanoic acid, trimethylsilyl ester	22.590	2.28
	Hexadecanoic acid, 2-oxo-, methyl ester	42.918	0.66
	Hexadecanoic acid, tetradecyl ester	44.807	1.02
4.	Stearic acid (C18:0)		
	Silane, (hexadecyloxy)trimethyl	20.494	0.54
	Octadecanoic acid, methyl ester	24.476	2.80
	Silane, trimethyl(octadecyloxy)	25.069	0.47
	Octadecanoic acid, trimethylsilyl ester	26.991	0.46
	Silane(1,1dimethylethyl)hexacosyloxydimethyl	40.217	0.13
	Silane, trimethyl(octacosyloxy)	44.196	0.39

Therefore, carbon chain length and degree of unsaturation are important characteristics for biodiesel production which may influence its properties and performance (Griffiths and Harrison 2009). Alternately, another study showed that increased accumulation of saturated fatty acid in biodiesel produced from a heterotrophic marine protist, *Schizochytrium sp.* PKU#Mn4, met the ASTM6751 standards (Wang et al. 2018). The above observations proved that saturated and unsaturated fatty acids exhibited by *R.toruloides* are desirable for biodiesel production and predicted to possess good flow property at low temperatures (due to unsaturated FAs) and is balanced by the presence of saturated FAs which resolve the oxidation problems caused by unsaturated FAs.

Physical and chemical properties

Properties and values obtained from transesterified lipid/biodiesel produced from *R. toruloides* have been tabulated in Table 2.

Physical properties

The lipid obtained had a faint fuel smell with slightly orange-brown color and a partially semi-solid (butter like consistency). However, after transesterification the state of lipid changed to semi-solid to liquid after reacting with alcohol and toluene.

Table 1 (continued)

5. Lignoceric acid (C24:0)			
Tetracontane	34.071	0.25	} 3.39
Tetracontane	35.906	0.16	
Tetracosanoic acid, methyl ester	36.535	0.49	
Tetracontane	39.445	1.20	
Tetracontane	43.211	1.29	
6. Cerotic acid (C26:0)			
Hexacosanoic acid, methyl ester	40.026	0.33	
Unsaturated fatty acid			
7. Palmitoleic acid (C16:1, Omega-7) [MUFA]			
9-hexadecenoic acid, methyl ester, (z)-	19.065	1.30	
8. Heptadecenoic acid (C17:1) [MUFA]			
cis-10-Heptadecenoic acid, methyl ester	21.934	12.67	} 13.54
cis-10-Heptadecenoic acid, methyl ester	21.978	0.16	
cis-10-Heptadecenoic acid, trimethylsilyl ester	24.551	0.71	
9. Oleic acid (C18:1, Omega-9) [MUFA]			
9-octadecenoic acid (z)-, methyl ester	23.954	3.79	} 17.59
9-octadecenoic acid (z)-, methyl ester	24.036	1.09	
9-octadecenoic acid, 12-hydroxy-, methylester	28.443	11.07	
Ricinoleic acid	28.583	1.08	
Trimethylsilyl (9z)-12-[(trimethylsilyl)oxy]-9-octadecenoate	30.601	0.56	
10. Linoleic acid (C18:2, Omega-6) [PUFA]			
Methyl 10-trans,12-cis-octadecadienoate	23.720	2.18	
11. Nonadecenoic acid (C19:1) [MUFA]			

Specific gravity

Specific gravity shows the density of a substance to that of water (1 g/cm³) at 15 °C. The specific gravity in our study was found to be 0.75 g/cm³ which is close to the standard range of 0.87–0.90 g/cm³; however, other ranges of specific gravity, i.e. 0.921–0.924 g/cm³ were considered in case of marine oils as their values were elevated as compared to the standard range reported (Ibeto et al. 2012 and; Kywe and Oo 2009). However, the higher viscosity of oils tends to cause

problems including incomplete combustion and particulate matter emissions, when used directly in diesel engines (Fort and Blumberg 1982; Ziejewski et al. 1993).

Water content

Water content in the biodiesel sample was found to be (900 ppm) 0.09% which was negligible and did not affect the properties of the sample being used as a fuel. The water content in oil must be within ASTM standards to

Table 1 (continued)

	cis-10-Nonadecenoic acid, methyl ester	26.500	11.28	
12.	Arachidonic acid (C20:4, Omega-6) [PUFA]			
	1-Eicosanol	47.409	0.51	
13.	Eurcic acid (C22:1, Omega-9) [MUFA]			
	13-Docosenoic acid, methyl ester, (z)-	32.302	0.22	
14.	Nervonic acid (C24:1) [MUFA]			
	3,5-Cyclo-6,8(14),22-ergostatriene	47.063	0.43	
15.	Cyclic aromatic compound			
	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	20.257	1.14	3.13%
	6H-[1,3]dioxolo[5,6]benzofuro C][1]benzopyran, 3-methoxy	38.304	1.81	
	4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-	45.669	0.18	

Table 2 Comparison of different physical and chemical properties of biodiesel produced from *R. toruloides* with other resources

Properties	Min/max value (ASTM standards)	<i>R. toruloides</i> biodiesel (this study)	Crude salmon oil (Dave et al. 2014)	Biodiesel from microalgae (Mostafa et al. 2017)	Jatropha curcus oil (Sarker 2016)
Physical state	–	Liquid	Clear liquid	Liquid	Liquid
Colour	–	Orangish-brown	Orange	Color index 1	–
Specific gravity	0.87–0.90 g/cm ³	0.75 g/cm ³	0.921 g/cm ³	0.868 g/cm ³	–
Water content	< 1500 ppm	900 ppm	325 ppm	39 ppm	700 ppm
pH	~7	9.5	6.8	9.24–9.13	7.8
Ash content	< 0.02%	0.03%	0.004%	Nil	4.2%
Free fatty acid	< 2.5%	4.17%	1.23%	–	–
Acid value	< 0.8 mg KOH/g	8.3 mg KOH/g	2.441 mg KOH/g	3.05 mg KOH/g	–
Refractive index	1.447–1.48	1.327	1.47	–	–
Calorific value	> 37 MJ/Kg	4254 Kcal/Kg	–	45.63 MJ/Kg	38.20 MJ/Kg
Iodine value	< 120 I ² /100 g	109 g I ² /100 g	116.79 g I ² /100 g	102 g I ² /100 g	94 g I ² /100 g
Cetane number	> 47	191.75 36.52	49.39	70	38

Figure 1 Separation of biodiesel and glycerol layers during transesterification of lipid produced from *R. toruloides*

Figure 2 TLC (thin layer chromatography) of lipid extracted from oleaginous yeast *R. toruloides* at different time points during its growth in waste extract

Figure 3 GC–MS (Gas chromatography- Mass spectrometry) analysis showing different fatty acid peaks of biodiesel produced from oleaginous yeast *R. toruloides*

prevent excessive soap formation during the chemical trans-esterification reaction which can lead to difficulties during separation of glycerol from ester (Wright et al.

1944). Generally, it is recommended that feedstock oil should not contain more than 1500 ppm of water to ensure successful transesterification.

Chemical properties

pH

The pH of the sample was recorded as 9.5. Devanesan et al. (2007) studied the effect of pH while producing biodiesel from *Jatropha* oil, using immobilized *Pseudomonas fluorescens* and reported an optimum pH value of 7.

Ash content

The residue left after the fuel is heated to sufficiently high temperature, also known as ash content, was found to be as low as 0.03%. The contaminants present in the fuel may lead to the formation of ash which may act as an abrasive and contribute to wear and tear. High amount of ash content may lead to sedimentation in fitting parts of fuel pumps and injector indicating that the fuel cannot be used for practical purposes (Ferrari et al. 2011; Nielsen 1998).

Free fatty acids (FFA) and Acid number

Free fatty acid indicates the quality of fats and oils and increases as the fatty acid breaks down into shorter chain acids (Mostafa and El-Gendy 2017). The free fatty acid content was found to be 4.17% and the acid value was also determined as high as 8.3 mg KOH/g (ASTM D664-664-9). It is reported that most effective conversion of triglycerides into esters during alkaline catalysis of transesterification occurs only if the free fatty acid level is less than 1% (Math et al. 2010); however, in this case we used acid catalysis process which did not impede biodiesel production. Pre-treatment may be used to decrease the acid number, if needed, for effective transesterification.

Refractive index

The refractive index of the sample was found to be 1.327 which indicates a lower degree of unsaturation. The refractive indices and peroxide values of the oils and fats significantly increase when exposed to light and heat (Arya et al. 1969). Generally oils' refractive index values vary between 1.447 and 1.482. The degree of unsaturation of oil greatly influences its oxidative stability and the stability of the biodiesel derived from it (Shahidi 2005).

Calorific value

It is the amount of energy released per unit quantity of the fuel on complete combustion. In our study calorific value of biodiesel was found to be 4254 Kcal/Kg as determined by the Bomb's calorimeter.

Iodine value

The iodine number gives an indication about the amount of unsaturated fatty acids (number of double bonds) in the oil and thereby indicates the ease of oxidation or drying capacity of the oil. In our study the iodine value was determined as 109 g I²/100 g which is within the range of ASTM standard (< 120 g I²/100 g). However, iodine value of oils are classified in different ranges: 125 g I²/100 g are classified as drying oils, iodine value 110–140 g I²/100 g are classified as semi-drying oils and iodine value less than 110 g I²/100 g are considered as nondrying oils (Ibeto et al. 2012).

Cetane value

The cetane number is used to measure the combustion quality of biodiesel and diesel fuels during compression ignition. Cetane number, in our study, was determined as 191.75 indicating higher degree of unsaturation. Bello et al. (2012) found the cetane number of cashew nut, egusi melon and rubber seed oils increased 10–40% after trans-esterification and the increase depended on the degree of unsaturation.

Discussion

While comparing biodiesel and fossil fuels, it was observed that the emission through combustion engines showed clear reduction in the following particulate: 48%-CO, 100%-SO₂, 67%-CO₂ and 47% particulate matter (Lotero et al. 2005). Therefore, biodiesel did not emit any reduced gas and showed zero net CO₂ and Sulphur emission, which are otherwise considered to be environmental pollutants (Antolin et al. 2002).

Biodiesel is produced by transesterification of fatty acid sources (e.g., TAGs) with short-chain alcohols yielding monoalkyl esters of long-chain fatty acids such as FAMES. To implement this process, microorganisms must be selected having the ability to produce fatty acids and short-chain alcohols available for transesterification. There are numerous oleaginous yeast, e.g. *Yarrowia lipolytica* that have been widely used to produce lipid and lipid-derived compounds such as biodiesel, edible oils or dicarboxylic acids as building blocks for polymers (Beopoulos et al. 2009).

The purpose of this study was to determine the properties of transesterified yeast lipid into biodiesel. *R. toruloides* was selected for its ability to produce significant amounts of TAGs, required for transformation into biodiesel. Total lipid was extracted from oleaginous yeast *R. toruloides* (cultivated in WE) and the lipid was analyzed by TLC to detect the presence of different fatty acids and TAGs. From the TLC plate, various kinds of components like TAG, free fatty acid

(FFAs), DAG were detected followed by quantification by GC–MS analysis.

Physical and chemical parameters for the transesterified lipid were performed to test its quality and compared to biodiesel derived from microbial and plant resources. It was noted that the Iodine value, in our study, was within the range, i.e. $109 \text{ I}^2/100 \text{ g}$, which should be $< 120 \text{ I}^2/100 \text{ g}$ according to ASTM standard, indicating reasonable degree of unsaturation that is desirable for biodiesel. Ash content in biodiesel produced by *R. toruloides* was recorded as 0.3% which nearly adhered to ASTM standard (0.02%) but lower than that from *Jatropha curcus* with higher (4.2%) ash content (Sarker 2016). Specific gravity was determined by ASTM D-287 standard and was recorded as 0.74 and 0.75 in diesel and biodiesel, respectively; however, they slightly deviated from ASTM standard values. Such deviations were also reported earlier wherein, Dave et al. (2014) demonstrated a lower value of 0.86, whereas, Mostafa et al. (2017) demonstrated a higher value of 0.92 in microalgae *Spirulina platensis* biodiesel. Refractive index value and calorific value in this study (1.327 and 17 MJ/Kg) were also relatively low with respect to ASTM standards i.e. (1.447–1.48 and $> 37 \text{ MJ/Kg}$) respectively. The water content of biodiesel should be $< 1500 \text{ ppm}$ according to ASTM standard, and in our study 900 ppm was recorded in the case of *R. toruloides*. Similar deviation was also reported by Sarker (2016) in which 700 ppm of water was found in biodiesel produced from *Jatropha curcus*. pH of biodiesel is a major factor for all types of transesterification, as the catalyst may be affected with very low pH value during the reaction. But pH value of biodiesel produced by *R. toruloides* was 9.5, which was relatively higher than 7 (ASTM Standard) but comparable to the values (9.24) recorded for microalgae *Spirulina platensis* biodiesel (Mostafa et al. 2017). Crude salmon oil exhibited a pH of 6.8 (Dave et al. 2014) and oil from *Jatropha curcus* showed pH as 7.8 (Sarker 2016). Free fatty acid value was found to be 4.17% in *R. toruloides*, that is, higher than ASTM standard (2.5%) and also crude salmon oil (1.23%) as shown by Dave et al. (2014). Acid value recorded in our study was found to be higher than ASTM standard ($< 0.8 \text{ mg KOH/g}$), and in microalgal and crude salmon oil, the acid value recorded was 3.05 mg KOH/g and 2.441 mg KOH/g, respectively, that is higher than ASTM (Mostafa et al. 2017; Dave et al. 2014). Higher acid values leads to problems in operating the engines due to corrosion and clogging. The acid value reported here is optimal and might be suitable for use in engines without causing corrosion damages.

The comparative analysis of various properties including specific gravity, pH, ash content, acid value, iodine value, *p*-anisidine value, peroxide value, TOTOX value, free fatty acid, flash point, kinematic viscosity, refractive index (Table 2) indicated that the biodiesel obtained from

yeast lipid is a suitable fuel and further removal of the water would enhance its fuel properties.

Conclusion

In this present study, biodiesel produced from *R. toruloides* was characterized for its physical and chemical properties and to determine its feasibility as a feedstock for biodiesel production. In the present study, the FAME profile and physico-chemical properties of biodiesel reported in this study showed that iodine value and ash content were within the range of ASTM standards. However, some parameters such as specific gravity, refractive index and calorific value were slightly lower than compared to ASTM standard and parameters, such as, the pH value, FFA, acid value and cetane number were higher than ASTM standard. Slight optimizations during biodiesel production may be implemented to enhance fuel properties.

Acknowledgements The funding by Dept. of Biotechnology (Govt. of India) is acknowledged by GS and DP. We also thank Dr. Abha Kumari, Mr. TP Pandey, Dr. Suresh Korpole (IMTECH) for necessary help and Dr. Paul (Maharaja Agrasen College, Delhi University) for her assistance in improving the language. Mr. Kashif M. Shaikh and Dr. Jutur form ICGB (Delhi) and the IARF facility at JNU are specially acknowledged for their help in TLC and GC–MS facility.

Author contributions All authors (GS, DP, CJ and KKB) contributed jointly to all the aspects of the work reported in the manuscript. GS performed most of the experiments and data analysis. DP and CJ co-supervised GS in this research. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human studies and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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