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Assessment of fuel properties on the basis of fatty acid profiles of oleaginous yeast for potential biodiesel production

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ABSTRACT

Over the last decade, there has been a huge upsurge of interest in sustainable production of biomass-based biofuels to fulfill the existing energy demand and simultaneously reducing the environmental deterioration. Earlier, vegetable oils and animal fats were utilized for biodiesel production, but due to food crisis and environmental sustainability, renewable sources such as neutral lipid derived from microbes are gaining much attention for budding biodiesel industries. Among various types of microorganisms, oleaginous yeasts are more promising feedstock to accomplish the current demand of biodiesel production and utilize a large number of cost-effective renewable substrates for their growth and lipid accumulation. However, biodiesel obtained from oleaginous yeasts have certain restrictions regarding their commercial utilization due to their unstable fuel properties such as oxidative stability, cetane number, viscosity and low-temperature performance etc. Numerous articles have been published in the public domain describing the fatty acid profiles of oleaginous yeast as feedstock for biodiesel production. However, the evaluation of quality parameters of biodiesel obtained from oleaginous yeasts is still in infancy. Although there is a huge disparity in a number of papers published for biodiesel production yet the reporting performance on diesel engines need to be verified in details. In this review article, attempt has been made to assess the important biofuel properties on the basis of the fatty acid profile of oleaginous yeast. Thus this evaluation would provide a guideline to the biodiesel producer to improve the production plans related to feedstocks for oleaginous yeast, culture conditions and biodiesel blending.

1. Introduction

Global energy threats have emerged due to robust population expansion, imbalanced food and fodder supply, reduction of fossil fuel reserves and receding natural resources. It is crucial to maintain sustainable and economical growth with the utilization of domestic and renewable sources of energy as to check the import of oils [1]. Among biomass-based biofuels, biodiesel is the most sustainable and renewable alternative to the fossil diesel fuel, well-defined as a blend of fatty acid alkyl esters [2–6]. It is chemically produced by transesterification reaction, in which triacylglycerides irrespective of its origin react with short chain alcohols (usually methanol/ethanol) to form alkyl esters [7,8]. This reaction is classified into two categories, catalyzed and non-catalyzed. Catalyzed transesterification process can be achieved by homogeneous, heterogeneous or enzymatic catalysts [9,10]. The most substantial procedure for transesterification reaction is using homogenous acid/base catalysts [11]. Sodium and potassium hydroxides (KOH/NaOH) as a base catalyst are used to convert the oil into fatty acid methyl esters (FAME) [12]. However, usage of the base catalysts has many critical issues such as saponification that causes the problem in separation and purification of the end product. Homogeneous catalysts are also very sensitive to free fatty acids (FFA) and water contents present in the oil. High FFA contents present in the feedstocks are responsible for soap formation when catalyzed with NaOH/KOH. In view of limitations associated with the homogeneous catalysts, solid heterogeneous catalysts for transesterification reaction are preferable due to their eco-friendly nature and the potential for producing purified biodiesel [13–16]. To combat these challenges there is a need for possible workout in the production of biodiesel through in-situ transesterification [17,18]. This process implies the direct use of the lipid-rich biomass without prior extraction of the lipids and allowing the transesterification reaction to take place within the solid matrix [17–20]. Biodiesel can be used for same conventional diesel engines regardless of its origin and feedstocks from which it is derived [21]. Low CO_2 emission without sulfur and aromatic contents are the important features that make it environment-friendly [21,22]. The usage of biodiesel is a sustainable practice to make our environment free from pollution and play a major role in the aspects of climate change as it

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Nomenclature

ACL	ATP-citrate lyase
ASTM	American Society of the International Association for Testing and Materials
CDW	cell dry weight
CFPP	cold filter plugging point
CFP	cold flow properties
CN	cetane number
D	density
DAG	diacylglycerol
DB	number of double bonds
DHAP	dihydroxyacetone phosphate
DU	degree of unsaturation
EU	European Union
FAME	fatty acid methyl esters
FC	% of each fatty acid component
G-3-P	glycerol-3-phosphate
h	hours
HHV	high heating value
HMF	hydroxyl methyl furfural
IS	Indian standard
IV	iodine value
KV	kinematic viscosity
LCSF	long chain saturation factor
LD	lipid droplets
M	molecular mass of each fatty acid component
MUFA	mono-unsaturated fatty acids
OS	oxidative stability
PA	phosphatidic acid
PAP	phosphatidate phosphatase
PHB	polyhydroxybutyrate
PUFA	poly-unsaturated fatty acids
SCO	single cell oil
SFA	saturated fatty acids
SV	saponification value
TAG	triacylglycerols
UFA	unsaturated fatty acids
UN	United Nations

contributes to no or little CO_2 in building up greenhouse gasses [23–27]. Generally, biodiesel is produced by transesterification reaction using edible vegetable oils [8]. Due to global food securities, oil derived from food sources cannot fulfill the requirement for large-scale biodiesel production and it is necessary to search novel non-edible renewable resources [28]. The use of animal fats, waste cooking oils, and oils from non-food crops as feedstocks are better alternative choices to reduce the production cost [29,30]. However, this strategy alone is not sufficient for the requirement of renewable fuels. Recently emphases have been shifted towards non-edible biomass-based biofuels due to decline in the availability of petroleum-based resources with increasing demand of energy [2]. In this regard, microbial sources for lipid production have many advantages over other sources which include short growth periods with higher lipid productivity deprived of any seasonal or climatic variations [31]. The major restraining issue for large-scale biodiesel production is high production cost specially related to microbial feedstocks, which can be reduced with the use of new cost effective bioprocess technologies [32]. Lipid produced by microorganisms, involving yeasts, bacteria, fungi and algae are called single cell oil (SCO) which is considered as promising feedstock for biodiesel production because of their similar fatty acid composition similar to vegetable oils [33–36]. These microorganisms utilize organic carbon and accumulate oil in the form of lipid droplets (LD) in their cellular compartments. The lipid productivity of several microorganisms has been reported more than that of oil-producing crops [37]. It has been reported that only a minor population of yeast accumulate more than 25% of lipids [37,38]. The species which are considered as oleaginous accumulate more than 60% of lipids such as *Rhodosporidium toruloides* 21167, *Rhodotorula toruloides* AS 2.1389, *Yarrowia lipolytica*, and *Cryptococcus curvatus*. Among these oleaginous yeast, *Rhodosporidium* spp. was able to produce the highest amount of lipid in their cellular compartment [33]. The culture of oleaginous yeast is neither affected by season nor by climate. In addition, oleaginous yeasts have specific property to accumulate lipids within its cellular compartments in a short duration which

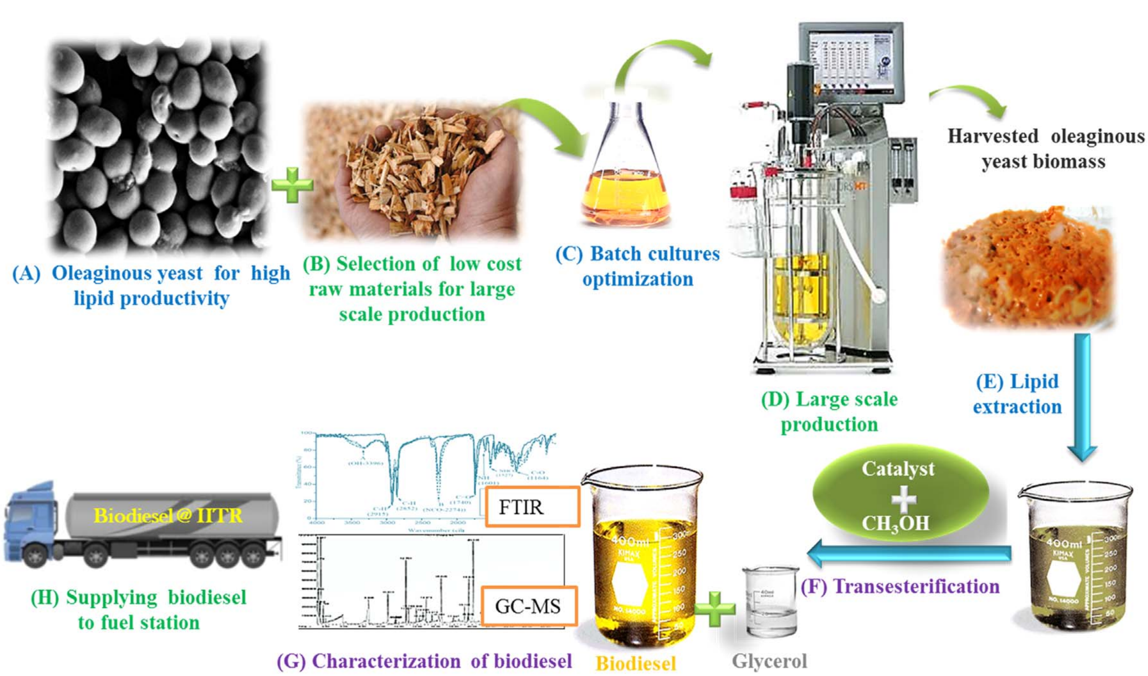

Fig. 1. Schematic diagram of biodiesel production from the oleaginous yeast.

varies from 5 to 9 days, depending on the species of yeast. The oleaginous yeasts have unique capability to utilize a large number of renewable substrates and inexpensive materials, such as agricultural and industrial wastes [39–43]. The idea to explore the non-edible lignocellulosic biomass as feedstock for oleaginous yeasts may greatly reduce the biodiesel production cost [7,44]. Moreover, the lipids accumulated by oleaginous yeasts chemically resemble with vegetable oil and animal fats. The relative composition of lipids in oleaginous yeasts was found to be $C_{18:1}$ (oleic acid) > $C_{16:0}$ (palmitic acid) > $C_{18:2}$ (linoleic acid)=$C_{18:0}$ (stearic acid) respectively [45]. Any alteration in the fatty acid profile influences the biodiesel properties during the transesterification process [46]. Interestingly, every microorganism possesses unique fatty acids profile depending on their growth conditions and feedstocks provided [47]. The quality of biodiesel is also influenced by the production process, refining process and post production parameters. Therefore, international standards namely ASTM 6751 (USA), IS 15607-05 (India) and EN 14214 (Europe) have been set up to monitor the quality and parameters of biodiesel [48]. The important parameters for potential biodiesel are viscosity (mm^2/s), oxidative stability (h), cetane number, cold filter plugging point (°C), density (kg/m^3), saponification value (mg KOH/g-oil), iodine value ($mgI_2/100$ g), and high heating value [49]. These biodiesel properties are majorly affected by compositional variations including fatty acids type, chain length, number and position of double bonds. Hoekman et al. proposed the correlation between unsaturation and biodiesel properties such as viscosity (mm^2/s), cold filter plugging point (°C), cetane number, iodine value ($mgI_2/100$ g), density (kg/m^3), and high heating value [48]. High saturation in fatty acid profile supports the CN, kinematic viscosity, and cold flow behavior while unsaturation in fatty acid profile supports the density and high heating value of biodiesel [50]. It has been observed by several researchers that combustion characteristic of fuel is also dependent on properties of particular biodiesel in which CN play an important role in engine performance [51]. Properties like density and heating value are directly correlated with CN. Due to higher oxygen content, biodiesel has higher CN which provides smoother engine operation [29]. CN of biodiesel varies according to different feedstocks utilized in the production of biodiesel. Researchers have shown that biodiesel possesses low viscosity than vegetable oil, so its flow rate is higher than other oils [52]. The main problem associated with biodiesel is low-temperature performance due to its high cold filter plugging point. Parameters like cold filter plugging point (CFPP), cloud point (CP), low-temperature filterability test (LTFT) which determines the cold flow behavior of diesel fuel and are also affected by the compositional changes in fatty acids [53]. Recently researchers have shown the impact of alcohol in improving the biodiesel properties [54]. This article summarizes the assessment of biodiesel obtained from various oleaginous yeast so as to develop a worksheet for biodiesel producer. The obtained parameters would help them to determine fuel blends that can minimize the risk of noncompliance with the technical requirements (Fig. 1).

2. Low-cost substrates utilized by oleaginous yeasts to produce lipids

To become more practical and continue to exist in the market, biodiesel must compete cost-effectively in order to compete with its counterpart. Industrial production of biodiesel still faces hurdles in terms of feedstocks availability and various steps involved in fermentation processes [55]. It has been observed that the feedstock accounts for 60–85% of the total cost of biodiesel, however, it can be substantially reduced if glucose based renewable substrates are used as carbon sources [56,57]. In order to further reduce the production cost, a substitute of the conventional process, in-situ transesterification is used where the conversion of oil into fatty methyl esters (FAME) is achieved directly from the wet biomass [58]. Ratledge and Cohen suggested that microbial oil is not yet a promising alternative for 2nd generation biodiesel production due to high production cost than the biodiesel obtained from vegetable sources. Even the disposability of de-oiled biomass generates several problems [59]. They supported their hypothesis by stating that within next 10–15 years when the price of vegetable oil will be too high than that of microbial oil will have a positive realistic market opportunity [59]. On the other hand, last decade had witnessed a huge expansion of interest in producing SCO amenable for biodiesel synthesis. This could be due to the interest in the microorganisms producing high quantities of essential edible lipids rarely found in the plant or animal kingdom i.e. lipids containing rare polyunsaturated fatty acids (PUFAs) or cocoa butter equivalents [60]. An alternative cost effective approach to the microbial oil for biodiesel production is to co-produce medically and dietetically important polyunsaturated fatty acids (PUFA) such as γ-linolenic, dihomo-γ-linolenic, arachidonic, and eicosapentaenoic acid [61–64]. Contrary to these assumptions, the recent scenario of biodiesel production witness cost effective pilot-scale biodiesel production of *Rhodosporidium toruloides* DEBB 5533 using a low-cost medium composed of sugarcane juice and urea [65]. They showed that the overall biodiesel production cost was economically competitive (US$ 0.76/l) to that of vegetable biodiesel (US$ 0.81/l) and the yield of biodiesel is 6.3-fold higher (4172 l/ha of cultivated sugarcane) than that obtained from yield of soybean biodiesel (661 l/ha of cultivated soybean) [65]. Besides this various low-cost raw materials such as sugarcane molasses (SCM) are extensively used as raw materials for oleaginous yeast due to its availability and high sugars contents [66]. It has been earlier reported that *Rhodosporidium toruloides* could produce 63.2% and 56.5% lipid content (w/w) when hydrolysates of non-edible crops Cassava and Jerusalem artichoke respectively were provided [67]. Several oleaginous yeasts such as *Cryptococcus curvatus*, *Rhodosporidium toruloides,* and *Yarrowia lipolytica* have also utilized hydrolysates of non-edible lignocellulosic biomass as a carbon source [28,68,69]. Sugarcane bagasse, sugar cane husk, wheat straw, rice straw and corn stover are the most promising non-edible lignocellulose biomasses/feedstocks in U.S.A, Asia, and Europe [70–72]. Recently, *Rhodotorula mucilaginosa* IIPL32 grown on the pentose fraction of acid pretreated sugarcane bagasse as a carbon source synthesized 15.3 g/l biomass along with 0.17 g single cell oil as per g of xylose consumed [73]. Certain oleaginous yeast such as *Cystobasidium oligophagum* JRC1 is capable of cellulase and lipase production simultaneously when grown on a wide range of substrates including carboxymethylcellulose (CMC) and accumulated 36.46% (w/w) lipid on the medium with CMC as sole carbon source [74]. Crude glycerol a byproduct of biodiesel industries too has been explored as a low-cost substrate by various oleaginous yeast for growth and lipid accumulation [31,75–77]. *R. toruloides* grown on crude glycerol produced 26.7 g/l cell dry weight with 70% intracellular lipid content [78]. *Rhodotorula glutinis* cultivated on pure and crude glycerol along with glucose as control showed highest lipid content (36.50%) with crude glycerol [79]. The production of biodiesel and obtainability of crude glycerol are a co-dependent process as increased production of biodiesel promotes the crude glycerol generation. Therefore, its utilization by oleaginous yeast has attracted much attention. *Trichosporon cutaneum and T. fermentans* grown in crude glycerol as carbon source produced 32.2% and 32.4% of total lipid respectively [80]. *T. oleaginosus* DSM 11815 cultivated on leftover of sweet sorghum juice from ethanol production accumulated 28% lipid content [81]. Similarly, *R. toruloides* AS 2.1389 produced 36.90 ± 4.36% lipid content when cultivated on reused medium of the non-sterile distillery and domestic mixed wastewater [82]. Another low-cost carbon source, acetic acid was utilized by *R. toruloides* AS 2.1389 which accumulated 48.2% lipid content with 4.35 g/l cell dry biomass [83]. When *C. curvatus* ATCC 20509 grown on acetate-rich corn stover hydrolysates, the cell dry weight, total lipid, and lipid content were 11.3 g/l, 6.9 g/l and 60.8%, respectively [84]. This strategy of co-fermentation of acetate and sugars by *C. curvatus* is promising for lipid production as the presence of acetate below 20 g/l promotes cell growth in terms of lipid productivity [84]. Oleaginous yeast *R.*

kratochvilovae HIMPA1 has unique ability to utilize pulp and paper industry effluent as a culture medium and accumulate high quantity of cell dry weight (13.87 g/l) with total lipid yield of 8.56 g/l within the cellular compartment [85].

3. Molecular studies on TAG synthesis in oleaginous yeasts

The molecular machinery involves in TAG synthesis by oleaginous yeast works in orderly and regulated fashion. Approaches generally employed in enhancing the lipid synthesis yield involve gene over-expression, the uses of knockout genes or multigene concept [86]. Oleaginous yeast can accommodate TAG as lipid droplets which may accounts for 70% of their biomass whereas non- oleaginous yeast such as *Saccharomyces cerevisiae* and *Candida ultilis* cannot accumulate lipid content more than 10% [87]. However, when they are grown in nitrogen-limited medium with excess carbon source, the contents of mannans and glucans increases in them, while in the case of oleaginous yeast the excess carbon source gets converted into lipids [88]. It has been reported that on nitrogen exhaustion from the culture medium, adenosine monophosphate deaminase in oleaginous yeast gets activated and catalyze the conversion of AMP into inosine 5′-monophosphate and ammonium as shown in Fig. 2 [89,90]. Further, the decreased concentration of AMP is responsible for the inactivation of isocitrate dehydrogenase leads to the destruction of metabolic pathway of tricarboxylic acid cycle [91]. In the cytosol of oleaginous yeast, ATP: citrate lyases (ACL) cleave the citrate and citrate translocate from mitochondria to cytosol via malate/citrate translocase system. Acetyl-Co-A formed in this reaction get converted into malonyl-Co A with the help of acetyl-Co A carboxylase enzyme. In the *de-novo* synthesis of lipids, both acetyl-Co A and malonyl-Co A add up to form fatty acid chains between 14 and 16 carbon long. Interestingly, absence of ACL in most of the non-oleaginous microorganisms has been found to be responsible for the synthesis of triacylglycerols as shown in Fig. 2b [88]. Furthermore, the fatty acid profile of oleaginous yeast is dependent on provided culture conditions as environment stress or physical stress [92,93]. Oleaginous yeast usually accumulates high lipid content in its cellular compartment in N-limited condition [94,95], while Gill et al., reported that *Candida* 107 can accumulate more lipid in phosphate-limited condition corresponding to high C/P molar ratio [96]. Similarly, Granger et al., observed that *Rhodotorula glutinis* accumulate maximum lipid under P-limited condition among various nutrients (N, P, Zn, Fe) limitation tested [97]. It has been suggested

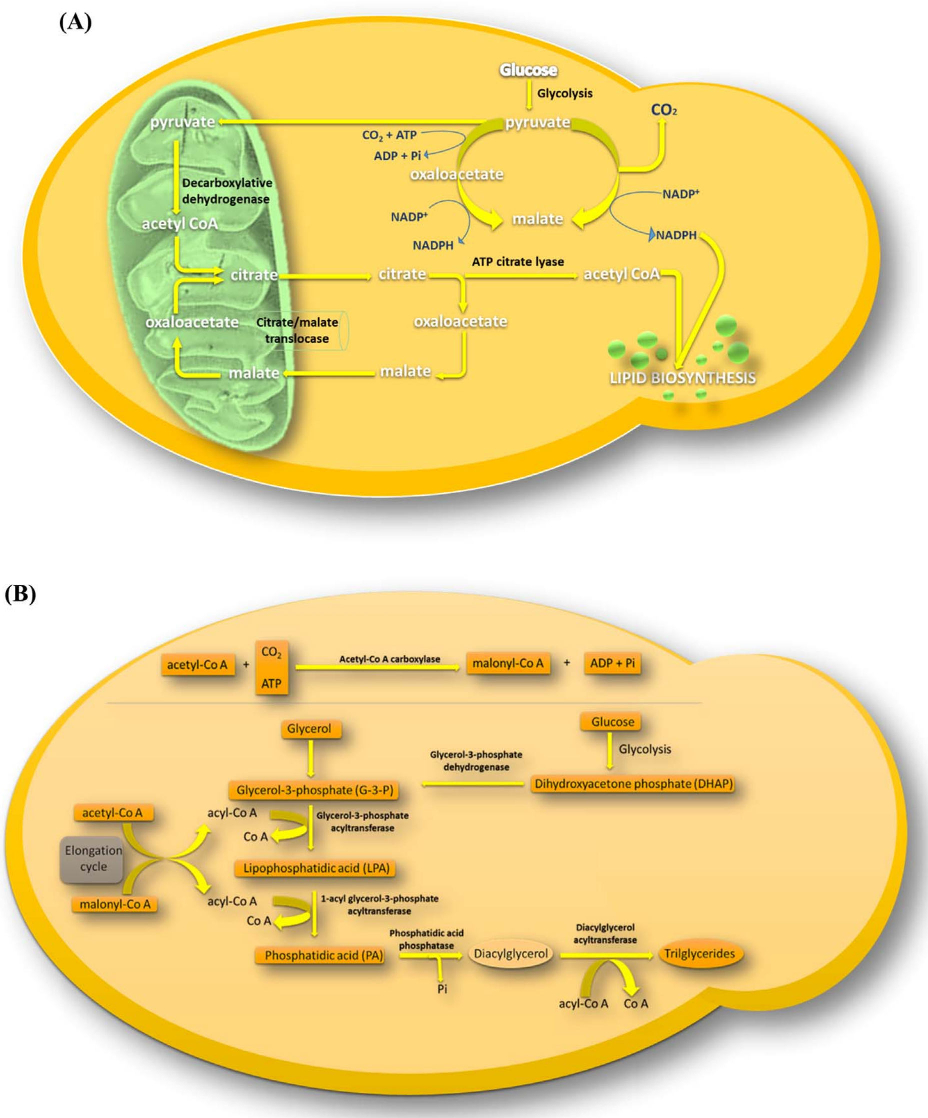

Fig. 2. (A) Schematic diagram of lipogenesis in oleaginous yeast and role of citrate and malate as precursors of acetyl-Co-A. (B) Role of malonyl-Co-A and acetyl-Co-A for TAG synthesis. Adapted and modified for reprinted with permission of Patel et al. [7].

that *R. glutinis* showed a small percentage of growth under N-limited condition and stopped growing when N was totally exhausted [98]. Also, it was observed that *Rhodosporidium toruloides* Y4, that accumulates high amount of lipid under P-limited condition [99]. However, the condition was absolutely dissimilar in phosphate exhausted condition where the culture show increased cell density and lipid-free biomass. Besides CNP ratio, temperature, pH, the presence of trace elements, aeration, and dissolve oxygen also affect the lipid accumulation in oleaginous yeast [35,100,101].

4. Fatty acid profile of oleaginous yeast grown on different substrates

Oleaginous yeast stores neutral lipids in the form of monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG) in their lipid bodies. Fatty acid profiles of different oleaginous yeasts are listed in Table 1. The oleaginous yeast species such as *Rhodosporidium toruloides* 21167, *Rhodotorula toruloides* AS 2.1389, *Yarrowia lipolytica*, and *Cryptococcus curvatus* have unique ability to synthesize $C_{14:0}$ (myristic acid), $C_{16:0}$ (palmitic acid), $C_{18:0}$ (stearic acid), $C_{18:1}$ (oleic acid), along with $C_{18:2}$ (linoleic acid). The fatty acid profile of these oleaginous yeast dependent upon the culture medium and various cultivation conditions provided as in case of *Rhodosporidium toruloides* Y4 which when grown in glucose synthetic medium with certain inhibitors (acetic acid, hydroxymethylfurfural, syringaldehyde, furfural, vanillin and polyhydroxy butyrate) showed variation in $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ content (Table 1). It has been reported that *Rhodotorula mucilaginosa* grown in 5-L airlift bioreactor can synthesized $C_{15:0}$ (3.4%), $C_{16:0}$ (20.2%), $C_{16:1}$ (1.2%), $C_{18:0}$ (4.3%), $C_{18:1}$ (42.6%), $C_{18:2}$ (27%), and $C_{18:3}$ (1.5%) when grown in the sea water [114]. Oleaginous yeast *Rhodosporidium diobovatum* grown on 100% pinewood pyrolysates accumulate mainly $C_{14:0}$ (0.8 ± 0.0%), $C_{16:0}$ (15.7 ± 0.4%), $C_{16:1}$ (0.9 ± 0.0%), $C_{17:0}$ (3.2 ± 0.1%), $C_{17:1}$ (3.7 ± 0.3%), $C_{18:0}$ (5.9 ± 0.6%), $C_{18:1}$ (49.2 ± 1.5%), $C_{18:2}$ (12.4 ± 0.9%), $C_{18:3}$ (0.8 ± 0.1%), $C_{24:0}$ (2.9 ± 0.4%) and 2.4 ± 0.1% other fatty acids [110]. In another study, *Cryptococcus curvatus* grown on synthetic media with 4 g/l acetic acid depicted fatty acid profile which includes $C_{16:0}$ (15%), $C_{18:0}$ (20%), $C_{18:1}$ (40%), $C_{18:2}$ (10%), similar to vegetable oil

Table 1
Fatty acids profiles of oleaginous yeast (OY) grown on different substrates.

S. no.	Oleaginous yeasts	Medium	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{22:0}$		References
1	*Rhodosporidium toruloides* 21167	Cassava starch	1.9	21.6	–	5.8	51.6	17.7	–	–	–		[102]
2	*C. curvatus* ATCC 20509	Volatile fatty acids	–	13.2	–	22.62	50.72	11.2	0.89	–	–		[103]
3	*Rhodosporidium toruloides* 2F5	Inulin	0.01	22.14	–	13.79	52.19	10.96	–	–	–		[104]
4	*Rhodosporidium toruloides* AS 2.1389	GSM	1.4	30.4	–	16.1	47.3	1.1	3.3	–	–		[105]
5	*R. toruloides* AS 2.1389	GSM	1.28	25.9	–	9.53	49.93	10.53	1.72	0.41	0.25		[106]
6	*Rhodosporidium toruloides*	Sodium lignosulphonate	1.6	27.5	3.1	11.9	44.2	9.5	2.3	–	–		[103]
7	*Rhodosporidium toruloides* Y4	GSM	1.8	33.8	0.5	13.4	48.3	1.1	–	–	–		[107]
8	*Rhodosporidium toruloides* Y4	GSM with Acetic acid (120 mM) Inhibitor	1.5	29.8	0.4	16	50.4	0.9	–	–	–		[107]
9	*Rhodosporidium toruloides* Y4	GSM with HMF (15 mM) Inhibitor	1.4	27.7	0.6	11.2	53.3	4.5	0.8	–	–		[107]
10	*Rhodosporidium toruloides 21167*	Hydrolysate of cassava starch	1.66	30.51	1.5	5.59	53.34	7.4	–	–	–		[108]
11	*R. toruloides*	Glucose	1.5	26.1	0	13	46.4	9.2	3.8	–	–		[78]
12	*R. toruloides*	Glycerol	1.6	28.7	0.2	15.3	41.5	10.1	2.6	–	–		[78]
13	*R. toruloides*	Glycerol with Sodium chloride impurity	1.2	25.9	0.9	12.1	46.3	11.7	2	–	–		[78]
14	*R. toruloides*	Glycerol with Methanol impurity	1.3	25.4	2.3	12.6	45.4	11.3	1.8	–	–		[78]
15	*R. toruloides*	Glycerol with Sodium oleate impurity	1.6	27.2	2.1	12.1	44.1	11.3	1.6	–	–		[78]
16	*R. toruloides*	Glycerol with methyl oleate impurity	1.7	25.2	3.2	10.8	43.1	13.4	2.6	–	–		[78]
17	*R. toruloides*	Glycerol with Glyceryl monooleate impurity	1.6	23.5	3.5	11	47.1	11	2.3	–	–		[78]
18	*Y. lipolytica*	Nondetoxified liquid wheat straw hydrolysate	–	6	–	2	56	19.9	–	–	–		[67]
19	*Y. lipolytica*	Detoxified liquid wheat straw hydrolysate	–	5.7	–	0.8	55.3	20.9	–	–	–		[67]
20	*C. curvatus*	Nondetoxified liquid wheat straw hydrolysate	–	25.9	–	15.2	47.7	6.42	–	–	–		[67]
21	*C. curvatus*	Detoxified liquid wheat straw hydrolysate	–	27	–	15.3	45	7.3	–	–	–		[67]
22	*R. glutinis*	Nondetoxified liquid wheat straw hydrolysate	–	23.5	–	9	43.4	15.4	–	–	–		[67]
23	*R. glutinis*	Detoxified liquid wheat straw hydrolysate	–	22.4	–	9.3	42.7	17	–	–	–		[67]
24	*Rhodosporidium fluviale* DMKU-RK253	Crude glycerol-YM (yeast extract- malt extract) medium	0.7	17.8		5.1	31.1	26.8	7.9				[109]
25	*Rhodosporidium toruloides* AS 2.1389	Acetic acid	1.08	19.29	0.35	16.28	44.51	10.57	3.32	0.59	0.00		[83]
26	*R. diobovatum* (08–225)	Pinewood pyrolytic sugars 100%	14.3	1.6		4.6	66.6	2.7			3.5		[110]
27	*Rhodosporidium fluviale* DMKU-SP314	Glucose and xylose	1.3	25.2	0.8	11.1	40.2	17.9	3.3				[111]
28	*Lipomyces starkeyi* ATCC 56304	Biphasic system sup- plying glucose for cell growth and xylose for oil production		21	3.1	6	64.7	3.1	0.9				[112]
29	*Rhodosporidium toruloides* DEBB 5533	Sugarcane juice	1	21.5	0.7	4.6	62.1	7.6	0.7	0.4	0.3	**$C_{24:0}$** 0.7	[65]
30	*R. toruloides* ATCC 10788	Crude glycerol media.		24.39		16.38	47.16	12.05					[113]
31	*R. kratochvilovae* HIMPA1	Aqueous extract of *Cassia fistula* L. (CAE) fruit pulp	0.78	43.06		28.74	17.34	0.48					[118]
32	*R. kratochvilovae* HIMPA1	Pulp and paper industry effluent		21.86		0.5	45.43	15.91		0.12			[85]

(–); Not detected.

composition but it get altered when pure volatile fatty acid (VFA) solution derived from waste activated sludge was used for lipid production [115]. They recorded that the percentage of fatty acids such $C_{15:0}$, $C_{17:0}$ and $C_{17:1}$, increased by 10, 38 and 53 times, respectively with decrease in contents of $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ when supernatant from anaerobically fermented waste activated sludge was used [115]. Studies on oleaginous yeast *R. kratochvilovae* HIMPA1 grown on various non-edible lignocellulosic biomass such as hemp seed aqueous extract, fermentable or non-fermentable carbon sources and *Cassia fistula* L. fruit pulp synthesized mainly myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid along with traces of linolenic acid [116–118]. When this oleaginous yeast was grown on Hemp seed aqueous extract (HSAE), the fatty acids contain mainly of $C_{16:0}$ (5.90%), $C_{18:0}$ (25.10%), $C_{18:1}$ (37.5%) along with $C_{20:0}$ (22%), $C_{22:0}$ (6.5%) and an unusual fatty acid $C_{27:0}$, (3%) [116]. While the fatty acid profile was changed when this oleaginous yeast was grown in non-edible lignocellulosic biomass of *Cassia fistula* L. fruit pulp [118]. Interestingly, when this oleaginous yeast was grown on pulp and paper industry effluent as a culture medium it synthesized high quantity of long chain monounsaturated fatty acid (45.43%) and polyunsaturated fatty acid (15.91%) that improves biodiesel quality under low temperature condition in terms of low CFPP along with good oxidative stability and cetane number as per ASTM D6751-02 and EN 14214 guidelines [85].

5. Assessment of biodiesel characteristics on the basis of fatty acid profile of oleaginous yeast

The biodiesel properties are totally dependent on the chemical constituent of used feedstock [119,120]. Fatty acid profile including chain length and the presence of unsaturation are an important factor in determining the physiochemical characteristics of biodiesel [54,121]. Biodiesel must meet the criteria set up by international standards such as ASTM 6751-3 (USA), EN 14214 (Europe) and Bureau of Indian Standard (IS 15607-05) for biodiesel [26]. The EN 14214 is implemented by all 31 associated states of the European Committee for Standardization [122]. Selected current specifications in the aforementioned two standards (ASTM 6751-3 and EN 14214) are listed in Table 2.

5.1. Long chain saturation factor (LCSF)

Long-chain saturated fatty acids are considered as the chains of carbon atoms that are completely saturated with hydrogen atoms and are of prime importance for determining the biodiesel quality [29,48,123]. High cetane number can be obtained with long chain saturated fatty acids in the feedstocks and can be correlated with reduced NOx emissions [124–126]. Low temperature or cold flow performance of biodiesel is determined by type and amount of saturated compounds in fatty acids [126]. The saturation in fatty acids is also a key player in determining the kinematic viscosity, where it increases with chain length and saturation. It can be calculated [48] with following empirical formula as shown in Fig. 3;

$$LCSF = (0.1 \times C_{16}) + (0.5 \times C_{18}) \quad (1)$$

The oil obtained from the oleaginous yeast *R. kratochvilovae* HIMPA1 grown in aqueous extract of *Cassia fistula* L. (CAE) fruit pulp showed highest amount of long chain saturated fatty acid (18.676) while *Y. lipolytica* grown in detoxified wheat straw hydrolysate and non-detoxified wheat straw hydrolysate exhibited the lowest amount of long chain saturated fatty acid (1.6 and 0.97 respectively).

5.2. Oxidative stability (OS)

Oxidative stability of biodiesel is an important yardstick to determine its self-life. Unsaturation and double bond in fatty acid chains are responsible for their interaction with oxygen when being exposed to air. It has been well documented that the degree of unsaturation, location and number of double bond severely affect the rate of autooxidation [48,126–128]. The multistep reaction of oxidative degradation is initiated with the generation of H atom from C which is adjacent to the double bond. Further, allylic hydroperoxides are formed with the reaction of oxygen after removal of H atom [128]. This is followed by secondary oxidative products which are formed by isomerization and radical chain propagation reaction. Researchers have stated that oxidatively unstable biodiesel decreases the engine performance due to high viscosity, the formation of gums and deposition of sediments [126,127]. Oxidative stability can be estimated by fatty acid profile with the help of following formula;

$$OS = 117.9295/(wt\% \quad C_{18:2} + wt\% \quad C_{18:3} + 2.5905) \quad (2)$$

Oxidative stability of biodiesel obtained from oleaginous yeasts is presented in Fig. 4. Fatty acids of *R. kratochvilovae* HIMPA1 grown in aqueous extract of *Cassia fistula* L. (CAE) fruit pulp showed maximum oxidative stability of 248 h while fatty acids obtained after growth in pulp and paper industry effluent as a culture medium showed the least oxidative stability (4.51 h).

5.3. Cold filter plugging point (CFPP)

An important consideration for biodiesel users is checked its performance at low temperature as gelling or crystallization in biodiesel at reduced temperature may severely affect the engine performance as it may clog the fuel line and filters [49,53,126]. Cold filter plugging point (CFPP) is the lowest temperature (°C) at which biodiesel easily passes through a standardized filtration device in a specific time [129–

Table 2
Selected technical specifications in the biodiesel standards ASTM D6751 and EN 14214 [1].

Biodiesel properties	Units	Biodiesel standard ASTM D6751		Biodiesel standard EN 14214	
		Test methods	Limits	Test methods	Limits
Oxidative stability,110 °C	h	EN 14112	3 h min	EN 14112, 15751	6 h min
Density	kg/m^3	–	–	EN ISO 3675, 12185	860–900
Cold filter plugging point	°C	–*	–*	–*	–*
Cetane number		D 613	47 min	EN ISO 5165	51 min
Viscosity	mm^2/s	D 445	1.9–6.0	EN ISO 3104, ISO 3105	3.5–5
Saponification value	mg KOH/g-oil	D 664	0.50 max	EN 14104	0.50 min
Iodine value	$mgI_2/100$ g	–	–	EN 14111	120 max
High heating value	–	–	–	–	–

– Not reported.
–* Cold filter plugging point with varying limits depending on geography and time of year.
Min=minimum.
Max=maximum.

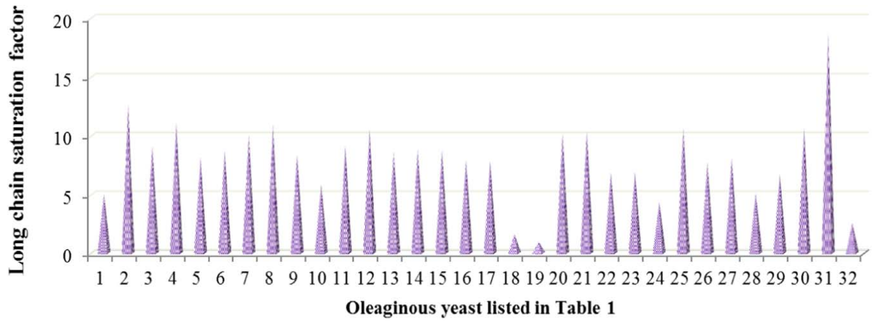

Fig. 3. Long chain saturation factor of FAME obtained from oleaginous yeast.

131]. CFPP can be estimated by following empirical formula;

$$CFPP = (3.417 \times LCSF) - 16.477 \quad (3)$$

where LCSF=Long chain saturation factor.

Among all listed oleaginous yeasts in Table 1, *Y. lipolytica* grown in detoxified liquid wheat straw hydrolysate and non-detoxified liquid wheat straw hydrolysate exhibited lowest CFPP of −13.16 °C and −11 °C respectively (Fig. 5). While the biodiesel derived from *R. kratochvilovae* HIMPA1 grown in aqueous extract of *Cassia fistula* L. (CAE) fruit pulp showed highest CFPP of 42.917 °C.

5.4. Kinematic viscosity (KV)

The property of viscosity of any fluid is just opposite to fluidity that repels the movement of fluid at intramolecular level [1]. Kinematic viscosity is an important fuel property of biodiesel that defined by its ability to flow, speed and quality of injected spray in the combustion chamber of the engine. The fluidity of biodiesel hampers as its viscosity increases at low temperature [29,50,120,132]. Viscosity increases with chain length of fatty acid or saturation of fatty acid, however, the viscosity of unsaturated fatty acid depends on number and nature of double bonds but less affected by position [133]. KV of biodiesel is usually 10–15% higher than the conventional diesel fuels due its large molecular weight and structure [133–135]. Ranges of KV specified by ASTM D 445 are 1.9–6.0 mm^2/s and 3.5–5.0 mm^2/s by EN ISO 3104. It can be calculated by the following formula;

$$\ln(KV) = -12.503 + 2.496 \times \ln(M) - 0.178 \times DB \quad (4)$$

where DB=double bonds, M=molecular mass of each fatty acid component.

Viscosity affects almost all components of diesel engines as it affects the starting of the engine, injection quantity and quality, and mixing of fuel with air in the combustion chamber. Viscosity having higher limit causes performance related problems especially at low temperature while lower limit causes fine particles of fuel with high speed and low mass. The data for kinematic viscosity show that all biodiesel types listed in Table 1 fall within a narrow range of 3.5–5 mm^2/s (Fig. 6).

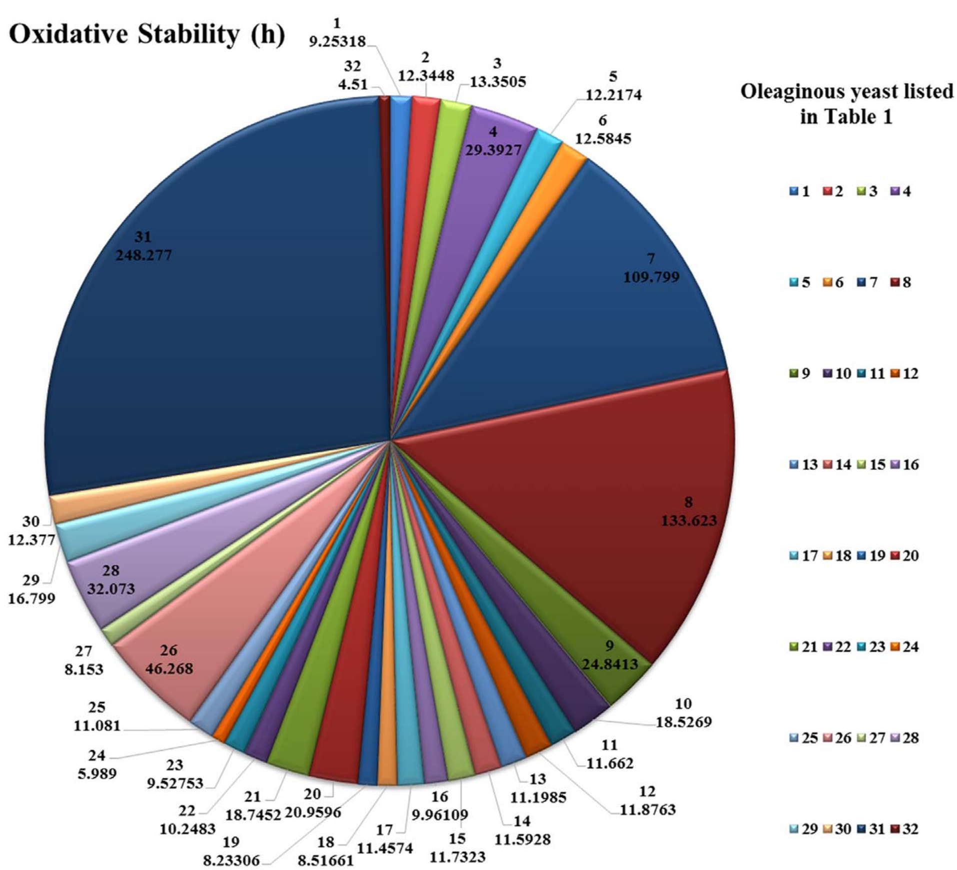

Fig. 4. Oxidative stability of oil obtained from oleaginous yeast grown on various substrates as listed in Table 1.

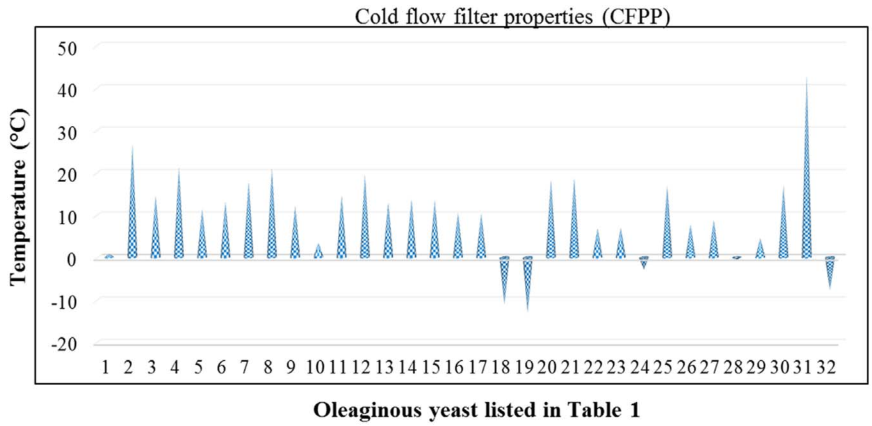

Fig. 5. Cold filter plugging point (CFPP) of oil obtained from oleaginous yeast grown on various substrates as listed in Table 1.

5.5. Density

Density play crucial role to determine the fuel injection property as it is correlated with another parameter for engine performance such as cetane number and heating value [48,50,136,137]. It affects the pumping of fuel by its volume and not so by mass [1]. It has been well documented that denser biodiesel has more energy than petroleum diesel [29]. Density is limited to 860–900 kg m^{-3} at 15 °C in EN 14214 but there is no specification for density in the ASTM D6751. The density of the fuel is correlated with other properties such as HHV, viscosity, cetane number which depends on temperature, water content, and the presence of free fatty acid content in FAME.

It can be calculated by the following formula;

$$Density = 0.8463 + 4.9/M + 0.0118 \times DB \tag{5}$$

The density of several oleaginous yeast oils mentioned in Table 1 is represented in Fig. 7. The oil obtained after *C. curvatus* ATCC 20509 utilized volatile fatty acids as the substrate has higher density (0.877379 g/cm^3) while the oil from *R. kratochvilovae* HIMPA1 grown in aqueous extract of *Cassia fistula* L. (CAE) fruit pulp showed the least density of 0.784 g/cm^3 among all listed oleaginous yeast in Table 1.

5.6. Saponification value

Saponification value defines the amount of KOH in mg required to saponify one g of fat under a specific condition and use to measure the molecular weight or chain length of fatty acids [138]. SV is usually low for long chain fatty acids due to a lesser number of carboxylic functional groups per unit fat mass than the short chain fatty acids [123,132]. SV can be calculated using following formula;

$$SV = 560(\%FC)/M \tag{6}$$

SV of oil obtained from *Y. lipolytica* grown in detoxified liquid wheat straw hydrolysate and non-detoxified liquid wheat straw hydrolysate showed the lowest amount of SV (160.054 and 157.757 mgKOH respectively) while *Lipomyces starkeyi* ATCC 56304 grown under the biphasic system (supplying glucose for cell growth and xylose for oil production) exhibited highest SV of 203.958 mgKOH as shown in Fig. 8.

5.7. Iodine value

Iodine value (IV) is the amount of I_2 in mg that is consumed by 100 g of substrates in a chemical reaction. It usually measures the addition of double bonds in fatty acids that are related to unsaturation [125,132]. European biodiesel standard, EN 14214 set the maximum value of 120 mgI_2/100 g for IV while it is not so well defined in ASTM D6751 [138].

Empirical formula for IV calculation;

$$IV = 254DB \times \%FC/M \tag{7}$$

IV of fatty acids obtained from oleaginous yeasts listed in Table 1 is presented in Fig. 9. Fatty acid obtained after *R. kratochvilovae* HIMPA1 grown in aqueous extract of *Cassia fistula* L. (CAE) fruit pulp showed the least amount of IV (16.462 mgI_2/100 g) while fatty acids obtained after growth in pulp and paper industry effluent as a culture medium showed highest Iodine value (120.017 mgI_2/100 g).

5.8. Cetane number (CN)

Cetane number (CN) is the property of fuel that decides the ignition characteristics of fuel in terms of ignition and combustion [126,137,139]. It affects the various parameters of engine performance such as noise, emissions of CO and stability [140]. Higher CN imparts the better ignition

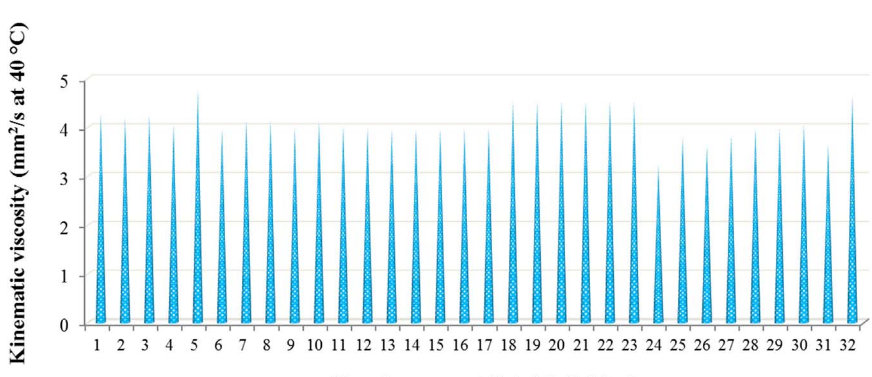

Fig. 6. Kinematic viscosity of oil obtained from oleaginous yeast grown on various substrates as listed in Table 1.

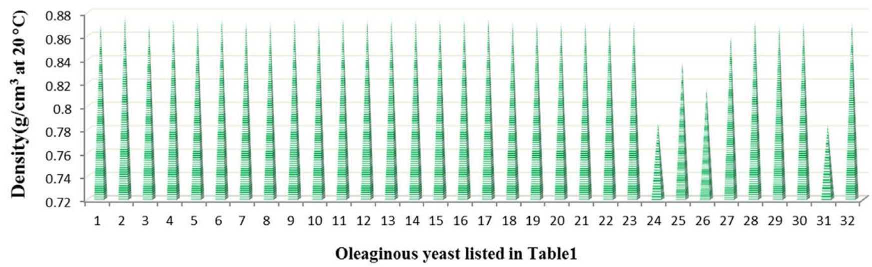

Fig. 7. The density of oil obtained from oleaginous yeast grown on various substrates as listed in Table 1.

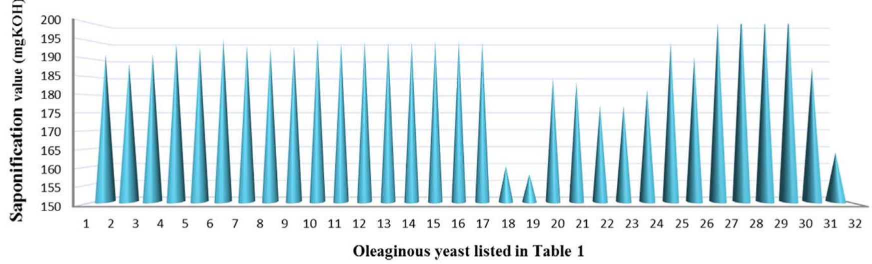

Fig. 8. Saponification value of biodiesel obtained from oleaginous yeast grown on various substrates as listed in Table 1.

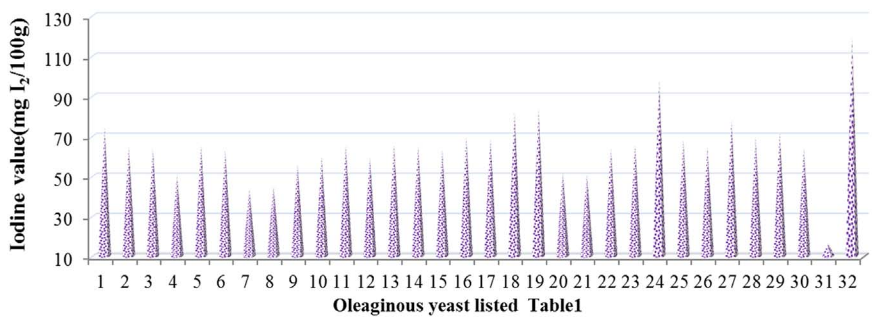

Fig. 9. Iodine value of biodiesel obtained from oleaginous yeast grown on various substrates as listed in Table 1.

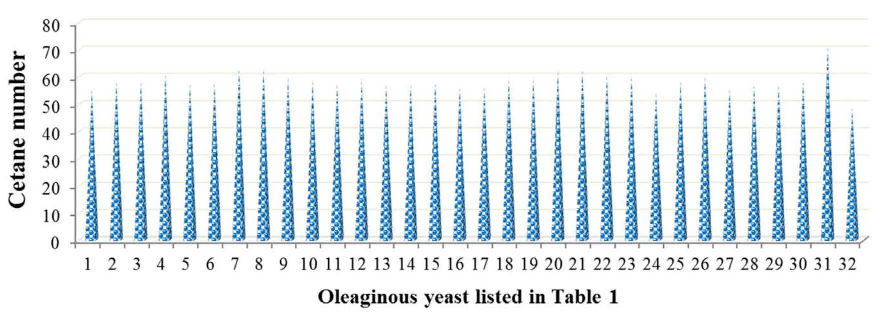

Fig. 10. Cetane number (CN) of biodiesel obtained from oleaginous yeast grown on various substrates as listed in Table 1.

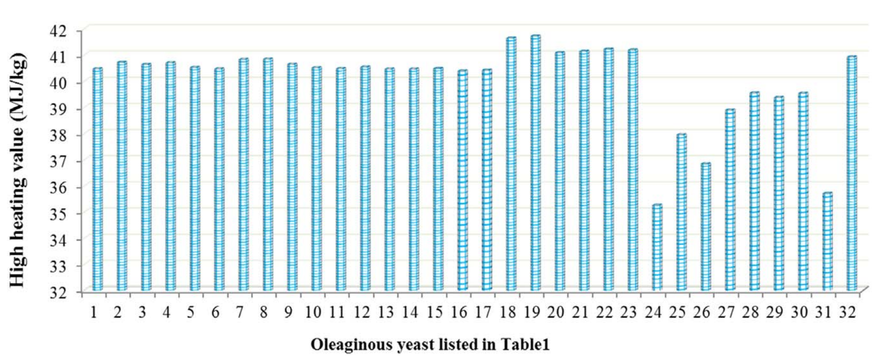

Fig. 11. The high heating value of biodiesel obtained from oleaginous yeast grown on various substrates as listed in Table 1.

of biodiesel than the conventional diesel fuel ensuring better cold start behavior, smooth engine run and complete combustion leading to reduced gaseous and particulate emissions [48,141,142]. Cetane number has both its lower and higher limits as lower cetane number of biodiesel causes difficulty of engine starting in cold environmental and generation of noise and pollution (emissions of hydrocarbons) without proper combustion of biodiesel while higher cetane number causes instant ignition without proper mixing of air that results in reduction of fuel efficiency. The fatty acid obtained after *R. kratochvilovae* HIMPA1 grown in aqueous extract of *Cassia fistula* L. (CAE) fruit pulp showed the highest amount of CN (71.649) while fatty acids obtained after growth in pulp and paper industry effluent as a culture medium showed least CN (49) the CN limit describe by both ASTM D6751-02 and EN 14214 (Fig. 10). CN of fatty acid methyl esters can be calculated by following empirical formula;

$$CN = 46.3 + 5458/SV - (0.255 \times IV) \quad (8)$$

5.9. High heating value (HHV)

The heating energy released during the combustion of the unit value of fuels is considered as the heating value of fuels and it is also known as calorific value or heat of combustion [143–146]. The elements of fuel such as O_2, H, C, N, and S after burning generates gaseous CO_2, NO_2, SO_2, and water along with heat. It is usually measured by bomb calorimeter according to ASTM-D2015 standard and with the help of fatty acid profile of feedstock by using following formula;

$$HHV = 49.43 - 0.041(SV) - 0.015(IV) \quad (9)$$

Both ASTM D6751 and EN 14214 standards do not have any specification for HHV. The fatty acids obtained from oleaginous yeast *Y. lipolytica* grown in detoxified liquid wheat straw hydrolysate and non-detoxified liquid wheat straw hydrolysate showed maximum HHV of 41.6312 and 41.7085 MJ/kg respectively (Fig. 11), while the least amount of HHV (35.246 MJ/kg) was obtained from *Rhodosporidium fluviale* DMKU-RK253 grown in crude glycerol-YM medium.

6. Conclusions and future outlook

The quality of biodiesel depends on the various parameters such as cetane number, cold filter plugging point, cold flow properties, viscosity, density, flash point, solidifying point and heating value etc. These properties are quite necessary for the determination of biodiesel potential as a substitute for diesel fuel. Even a single change in fatty acid profile severely affects the biodiesel properties. Factors mainly fatty acid chain length, unsaturation, number and position of double bonds are considered to be prime importance to make biodiesel as promising alternative fuel. The presence of high SFA (saturated fatty acids) in FAME mitigates the biodiesel to undergo auto-oxidation and thereby increasing its shelf-life while UFA (unsaturated fatty acids) quantities determine its cold flow plugging properties. Hence, it is necessary to control the fuel properties by optimizing the ratio of SFA to UFA. Researchers have shown that unsaturation in fatty acids leads to higher CFPP with poor oxidative stability specially in the case of *Y. lipolytica* grown in detoxified liquid wheat straw hydrolysate and non-detoxified liquid wheat straw hydrolysate. It showed high CFPP and HHV, while OS and SV were drastically reduced. Parameter such as KV majorly affects the injection of fuel in the engine as higher viscosity leads to larger droplet sizes, low vaporization, and reduced injection spray angle. According to survey conducted in this article, the biodiesel obtained from oleaginous yeast have some lacuna regarding its properties related to poor oxidation stability and cold flow property. These parameters are responsible for the production of harmful oxidation products under extended storage periods and can clog the fuel pipeline in cold weather conditions. To combat these problems blending of biodiesel with petroleum diesel fuel is a desirable choice. However, care should be taken regarding the biodiesel concentration that should be lower in the blends as increased concentration of biodiesel in the blend can cause increment in carbon residue, viscosity and cold flow properties (CFP), which remarkably affect the fuel flow system and combustion process. The problems associated with CFP can be resolved by using the additives such as polymethyl acrylate (PMA). The biodiesel properties such as cetane number, oxidation stability, iodine value, density and viscosity also fluctuate according to their regional variations reflected by weather conditions. Therefore, establishing strategies regarding uniform formulation guidelines of biodiesel needs to be designed that majorly affects its large-scale imports and exports among different regions of the world.

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