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Research Article

Microbial oil production from various carbon sources by newly isolated oleaginous yeasts

Microbial oil production has received significant attention as a potential precursor for the production of biofuels, oleochemicals and food products. In this study, six oleaginous yeasts, isolated from fruits, were selected based on their ability to accumulate high intracellular content of microbial oil (20–48% w/w of total dry weight). The highest content of saturated fatty acids was 68.7% (w/w), whereas the highest content of oleic acid was 62.7% (w/w). Furthermore, nutrient-rich hydrolysates produced via enzymatic hydrolysis of flour-rich waste streams generated by a confectionery industry were evaluated as fermentation media for microbial oil production via fed-batch bioreactor cultures using one of the most promising isolates, namely VV_D4. A total dry weight of 40 g/L with a microbial oil content of 39% (w/w) was produced by isolate VV_D4. Critical biodiesel properties were estimated based on the fatty acid composition and correlated with the international standards. The microbial oil produced by the new isolates could be potentially used for biodiesel production.

Keywords: Biodiesel / Biorefineries / Fermentation / Microbial oil production / Yeast isolation

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1 Introduction

Microbial oil is commonly defined as lipids that are accumulated by oleaginous microorganisms. In general, oleaginous microorganisms are able to accumulate intracellular oil at more than 20% of their cell dry weight. Many microorganisms that belong to the genera of yeast, fungi, bacteria and algae have been reported to accumulate significant amounts of lipids during fermentation on various renewable resources [1]. Among these, oleaginous yeasts are desirable industrial microorganisms due to their ability to accumulate high lipid content (up to 70% w/w), achieve high growth rates, tolerate high sugar concentrations, and utilize a wide range of carbon sources. Several strains belonging to the genera *Rhodosporidium* sp., *Rhodotorula* sp., *Yarrowia sp.*, *Cryptococcus* sp., *Lipomyces* sp., have been reported to accumulate lipids between 40 and 70% of their dry cell weight under nutrient-limiting conditions [2].

Microbial oil accumulation in yeasts fulfills energy storage purposes and it consists of triaglycerols (TAGs) which are produced in the endoplasmic reticulum and lipid bodies in the cytosol. Oleaginous yeasts are able to utilize a wide range of carbon sources for *de novo* lipid biosynthesis. Microbial oil production is triggered under nutrient limiting conditions leading to accumulation of citric acid in the mitochondrion that is subsequently secreted into the cytoplasm where it is converted to acetyl-CoA (the precursor for fatty acid synthesis) and oxaloacetate by ATP citrate lyase [1]. Various substrates can be valorized by oleaginous yeasts for microbial oil production such as pure sugars, sugar-rich wastes (molasses and cheese whey), lignocellulosic materials, biodiesel derived glycerol, starch and food-processing wastes. The food industrial sector produces huge amounts of by-product streams. Confectionary industrial residues mainly consist of starch and other nutrients that are required for microbial growth (e.g. proteins, minerals) [3]. Lignocellulosic biomass could be also used for microbial oil production. Complete hydrolysis of lignocellulosic materials produce a variety of monosaccharides that consist of pentoses (xylose, arabinose) and hexoses (glucose, galactose, and mannose), but also inhibitors (furfural, HMF, acetic acid) are generated [4]. The utilization of all sugars derived from agri-industrial waste streams is essential in order to enhance the microbial oil production via fermentation [1].

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Abbreviations: BLAST, basic local alignment search tool; **CFPP**, cold filter plugging point; **CN**, cetane number; **FAMEs**, fatty acid methyl esters; **FAN**, Free amino nitrogen; **FID**, flame ionization detector; **FRW**, flour-rich waste; **GenBank**, genetic sequence database; **HMF**, 5-hydroxymethyl-furfural; **HPLC**, high performance liquid chromatography; **IPS**, intra-cellular polysaccharides; **LC**, length of chain; **LCV**, low calorific value; **PCR**, polymerase chain reaction; **rRNA**, ribosomal ribonucleic acid; **TDW**, total dry weight; **YPD**, yeast extract peptone dextrose

Microbial oil production by oleaginous yeasts can be used for biodiesel production and as a raw material for oleochemical production such as detergents, soaps, plastics, surfactants, lubricants, paints, and additives for the food and cosmetic industry among others [1,5]. Biodiesel is a renewable, biodegradable and non-toxic energy resource that can be produced by transesterification of triacylglycerols (from edible and non-edible oil-rich feedstocks) with methanol to produce fatty acid methyl esters. However, due to the continuously increasing demand for first generation biodiesel and the fact that biofuel production should depend on non-edible feedstocks, researchers focus on the production of microbial oil as an alternative feedstock for biodiesel production.

Microbial oil produced by oleaginous microorganisms has unique fatty acid composition which is affected by the type of carbon source, nature of microorganism and the cultivation conditions. In most cases, the fatty acid profile is similar to plant and animal oils, containing mainly C16 and C18 fatty acids. To evaluate the suitability of biodiesel production from microbial oil, several properties should be analyzed. Cetane number, kinematic viscosity, flash point, low calorific value, cold filter plugging point are some of the physicochemical biodiesel properties that must conform to the international standards (American Society for Testing and Materials ASTM D6751 and EN 14214 in Europe). Many researchers have developed mathematical models in order to predict the dependence between the fatty acid composition and biodiesel properties [6,7].

Another potential application of the microbial oil produced by yeast strains in the food industrial sector could be the production of exotic fats such as cocoa butter substitutes. Cocoa butter has high saturated fatty acid content (approximately 60% and particularly 35% stearic acid and 25% palmitic acid). Papanikolaou et al. evaluated single cell oil production by *Yarrowia lipoytica* that could be used as substitute for cocoa butter [8].

In this study, several yeast strains were isolated from different fruits and studied for lipid accumulation utilizing sucrose as carbon source. Six isolates that indicated high level of intracellular lipid content were selected and identified. Subsequently, the utilization of various carbon sources for lipid production by the selected yeast strains was studied. The most promising isolate, VV_D4, was used in order to conduct a fed-batch bioreactor fermentation using hydrolysates from flour-rich waste streams as fermentation media. The fatty acid composition was determined and the physicochemical properties of each microbial oil were estimated using literature-cited mathematical models in order to evaluate the potential of these oils for biodiesel production.

2 Materials and methods

2.1 Isolation

The yeast strains were isolated from the surface and the body of red and white grapes (*Vitis vinifera*), apples (*Malus domestica*), prickly pears (*Opuntia ficus-indica*), pears (*Pyrus communis* and *Pyrus pyrifolia*), damsons (*Prunus domestica subsp, insititia*), plums (*Prunus domestica*), melons (*Cumunis melo*), pomegranates (*Punica granatum*) and figs (*Ficus carica*). 10 g of the samples were suspended in 90 mL of sterile saline solution and shaken vigorously in a stomacher apparatus (Laboratory Blender 400, Seward Medical, London) for 60 seconds. Serial dilutions of the sample suspension were performed in sterile saline solution. An amount of 0.5 mL was spread on Yeast Extract Dextrose Chloramphenicol agar (LAB M, Lancashire, UK) and incubated for 24 –48 h at 28°C. After incubation, morphologically different colonies were selected and isolated by successive subcultures on Yeast Extract Dextrose Chloramphenicol agar. Then, each colony was transferred with a wire loop into liquid media containing (in g/L): 20, sucrose; 0.3, yeast extract; 0.5, corn steep liquor; 11.7, $NaH_2PO_4 \cdot 2H_2O$; 1.5, K_2HPO_4; 1, NaCl; 1, $CaCl_2 \cdot 2H_2O$; 0.04, $MgSO_4 \cdot H_2O$; 0.044, $ZnSO_4 \cdot 7H_2O$; 0.016, $FeCl3 \cdot 6H_2O$ in a 100 mL Erlenmeyer flask with 20 mL total fermentation volume and incubated for 24–48 h at 180 rpm and 28°C. The yeast strains which showed lipid globules within the cell were selected for the screening study.

2.2 Screening of oleaginous yeasts

For the selection of the oleaginous yeasts, isolates that indicated lipid accumulation were initially cultivated in 250 mL Erlenmeyer flasks, containing 50 mL of the liquid medium presented above. Cultures were inoculated with 10% (v/v) of 24-h preculture inoculum in YPD medium (10 g/L glucose, 10 g/L peptone, 10 g/L yeast extract). Flasks were incubated in an orbital shaker at an agitation speed of 180 rpm for 48 h and 28°C. The initial pH was adjusted to 5.5 before autoclaving.

2.3 Screening of the selected strains

The selected yeast strains were further cultivated in 500 mL Erlenmeyer flasks containing 100 mL of the growth medium presented above. The carbon sources used were glucose, xylose, mannose, arabinose and galactose with initial concentration of 20 g/L. Also a control without the carbon source was used so as to determine the total dry weight (TDW) produced from nitrogen sources and minerals only. Cultures were inoculated with 10% (v/v) of 24-h pre-culture inocula. The pre-culture used was the same as described above. The shake flask fermentations have been carried out in triplicates.

2.4 Identification of oleaginous yeast strains

DNA extraction and amplification of the D1/D2 region of 26S-rRNA gene were performed according to Pateraki et al. [9]. PCR amplification products were sent to a commercial facility for sequencing. The results were aligned with yeast type strains in GenBank using the BLAST program to determine the closest known relatives based on the partial 26S-rRNA gene sequence.

2.5 Production of flour-rich waste hydrolysate

A generic feedstock from flour-rich waste (FRW) hydrolysate was produced using the optimized method reported by Tsakona et al. [3]. In brief, crude enzymes produced by solid state fer-

Table 1. Mathematical models to predict the properties of microbial oil derived biodiesel

Properties	Unit	Equation model	R^2	References
Unsaturation degree	–	$UD = (1\%_{MU} + 2\%_{DU} + 3\%_{TU})/100$	nr	[12]
Chain length	–	$LC = R(nC_n\ c_n)$,	nr	
Cetane number	–	$CN = \Sigma\ X_{ME}\ CN_{ME}$	nr	[7]
Cold filter plugging point	°C	$CFPP = -0.4880X+36.0548\ (0<X\leq 88)$	nr	
		$CFPP = -2.7043X+232.0036\ (88<X\leq 100)$		
Low calorific value	kJ/kg	$LCV = 29385.4 + 486.866\ LC - 387.766\ UD$	98.22	[12]
Flash point	°C	$FP = 1008.48 - 136.166\ LC + 142.578\ UD + 5.14811\ LC^2 - 10.6906\ LC\ UD + 9.26352\ UD^2$	95.94	
Viscosity	mm^2/s	$\mu = -1.8327 + 0.209794\ LC + 0.738911\ UD + 0.0166791\ LC^2 - 0.16336\ LC\ UD + 0.335547\ UD^2$	96.69	

nr: not reported
Where nC_n is the number of carbon atoms of each fatty acid, c_n is the percentage of weight of each methyl ester containing this fatty acid, $\%_{MU}$ is the percentage of weight of monounsaturated methyl esters, $\%_{DU}$ is the percentage of weight of di-unsaturated methyl esters and $\%_{TU}$ is the percentage of weight of tri-unsaturated methyl esters, X_{ME} is the weight percentage of each methyl ester and CN_{ME} is the cetane number of individual methyl ester, X is the content of the unsaturated fatty acid (wt %)

mentation of *Aspergillus awamori* were mixed with a suspension of flour-rich waste streams for the production of fermentation feedstock that was subsequently evaluated in fed-batch experiment for microbial oil production by the isolate with code name VV_D4. The initial flour concentration was 100 g/L. The solid state fermentation was carried out in 250 mL Erlenmeyer flasks using 5 g of wheat milling by-products. This process is described in detail by Tsakona et al. [3].

2.6 Bioreactor fermentation

The fed-batch fermentation was conducted in a 3.4 L bioreactor (Labfors 4, Infors HT) with a working volume of 2 L using FRW hydrolysate as a carbon and nutrient source supplemented with yeast extract and corn steep liquor in order to achieve an initial FAN concentration of 260 mg/L. The mineral content added in the fermentation medium was (in g/L): 11.7, $NaH_2PO_4{\cdot}2H_2O$; 1.5, K_2HPO_4; 1, NaCl; 1, $CaCl_2{\cdot}2H_2O$; 0.04, $MgSO_4{\cdot}H_2O$; 0.044, $ZnSO_4{\cdot}7H_2O$; 0.016, $FeCl3{\cdot}6H_2O$. The pH was maintained at 5.7 by automatic addition of 5 M NaOH and 10% (v/v) H_2SO_4. The air flow rate was maintained at 1 vvm and the temperature at 27°C. The agitation rate was controlled in the range of 150–400 rpm aiming to maintain the dissolved oxygen (DO) concentration at 20% of saturation. A 10% (v/v) of inoculum cultivated for 24 h was used. The initial glucose concentration was 10 g/L and feeding addition began when glucose concentration reached 5 g/L. A concentrated glucose solution (600 g/L) was added in the bioreactor in order to maintain the concentration of glucose at the range of 5–25 g/L. Fermentation samples were taken periodically for the analysis of glucose, free amino nitrogen (FAN), total dry weight, total intra-cellular polysaccharides (IPS) and intracellular lipids.

2.7 Analytical methods

2.7.1 *Determination of total dry weight*

Each sample of fermentation broth (10 mL) was harvested by centrifugation at 9000 × *g* for 10 min (Hettich Universal Centrifuge, model 320-R, United Kingdom) and washed twice with distilled water. The total dry weight was determined gravimetrically after drying at 102°C for 24 h.

2.7.2 *Determination of sugars, intracellular polysaccharides and free amino nitrogen*

Sugar concentration was determined by an HPLC SHIMADZU UFLC XR system equipped with a Phenomenex Rezex ROA column with size 300 mm × 7.8 mm coupled to a differential refractometer. Operating conditions were as follows: sample volume 20 μL; mobile phase 10 mM H_2SO_4; flow rate 0.6 mL/min; column temperature 45°C for the determination of sucrose and glucose, while 70°C was used for galactose, mannose, arabinose and xylose determination.

Table 2. Total dry weight, lipid concentration and lipid content achieved by the selected oleaginous yeast strains in shake flask fermentations using sucrose as carbon source

Isolation source	Code name	Time (h)	Total dry weight (g/L)	Lipid concentration (g/L)	Lipid content (% w/w)
Prunus domestica	PD_D2	48	8.0 ± 0.30	2.8 ± 0.31	34.8 ± 1.57
Prunus domestica	PD_F1	48	7.3 ± 0.10	2.0 ± 0.11	27.3 ± 1.88
Vitis vinifera	VV_D4	48	8.0 ± 0.17	2.7 ± 0.06	33.2 ± 0.12
Pyrus communis	PC_A2	24	5.6 ± 0.17	2.7 ± 0.28	48.6 ± 1.48
Pyrus pyrifolia	PP_D3	48	8.4 ± 0.31	2.1 ± 0.09	25.0 ± 0.10
Malus domestica	MD_F1	48	8.0 ± 0.06	2.5 ± 0.20	31.9 ± 2.27

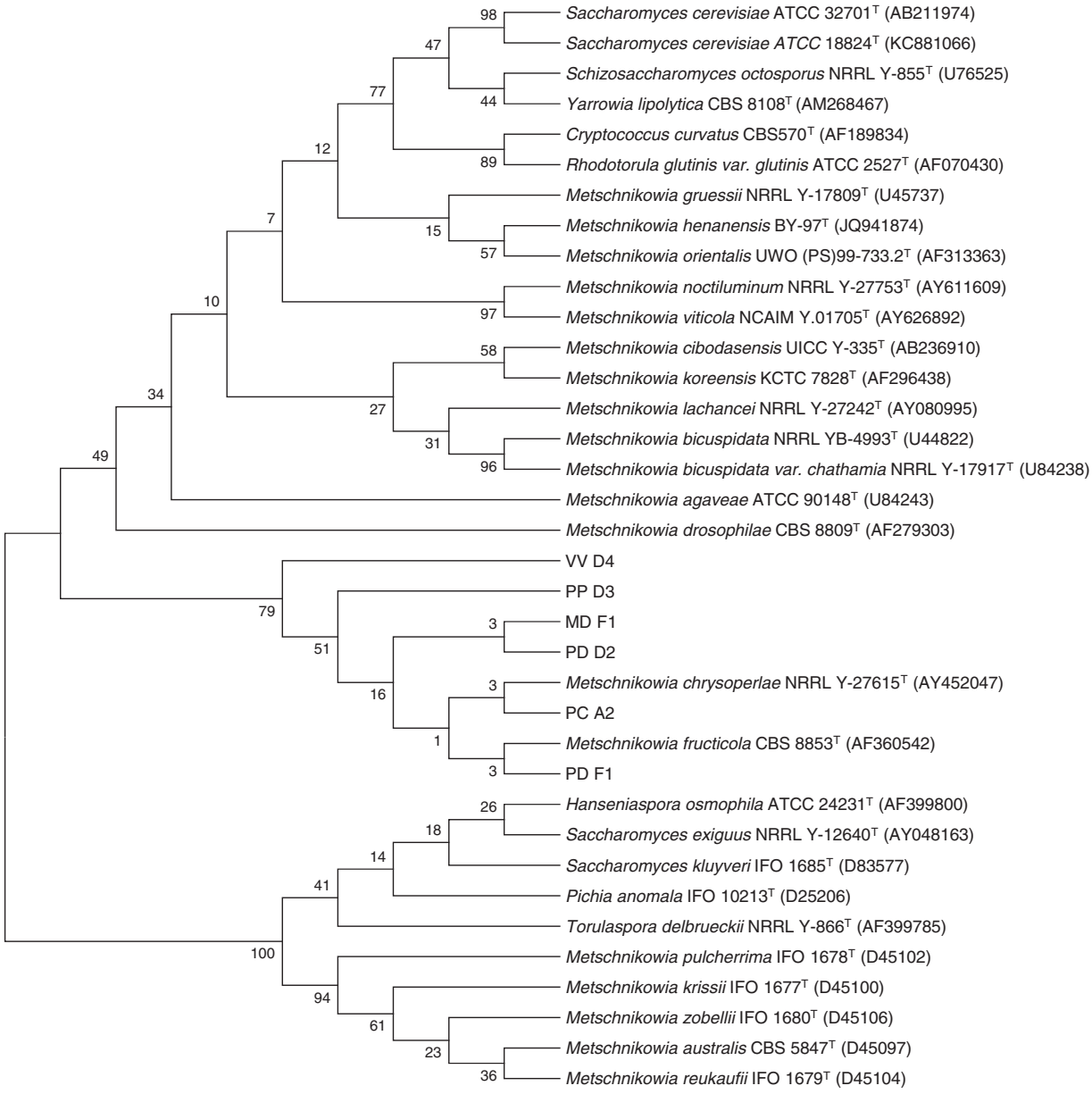

Figure 1. Phylogenetic position of the newly yeast isolates and comparison with type yeast strains based on Neighbor-Joining distance analysis of 26S rRNA gene sequences. Bootstrap values at the node were calculated from 1000 samplings. GenBank/EMBL/DDBJ accession numbers are given in parenthesis.

Total IPS was determined by the method reported by Tsakona et al. [3] with slight modifications. Briefly, 2 mL of hydrochloric acid (2 M) were mixed with 50 mg of dry weight of yeast cell mass followed by boiling (100°C) for 30 min. Cellular debris was removed by centrifugation and the supernatant was analyzed by HPLC for IPS determination. FAN concentration was quantified according to the ninhydrin colorimetric method as reported by Lie [10].

2.7.3 Determination of lipid content

The lipid content was extracted and determined according to Folch et al. [11] with few modifications. An acid pretreatment method was performed by adding 4 mL of hydrochloric acid (2 M) for each 300 mg of dry cell mass weight and incubating at 80°C for 1 h. Then, the acid hydrolyzed mass was centrifuged (6000 × g for 10 min) to separate the aqueous upper phase from the organic lower phase. The lower phase containing the

Table 3. Phylogenetic position of the isolates based on sequencing of the D1/D2 region of 26S-rRNA gene

Strain	Closest relative	Identity (%)	Accession number
PD_D2	*Metschnikowia sp.* 11–1088 clone e2	98%	KM275357
VV_D4	*Metschnikowia aff. pulcherrima* P01A016	98%	JX188181
PC_A2	*Metschnikowia pulcherrima* strain B-NC-13-F08	99%	KJ794649
PD_F1	*Metschnikowia pulcherrima* isolate Qch1	99%	HM067867
PP_D3	*Metschnikowia pulcherrima* strain BZYXYL-1	97%	JX041890
MD_F1	*Metschnikowia aff. pulcherrima* P01A016	99%	JX188181

lipids was stirred with chloroform: methanol mixture (2:1). Total cellular lipid was recovered and gravimetrically quantified by Folch et al. [11].

2.7.4 Fatty acid methyl-esters (FAMEs)

Lipids were converted to methyl-esters according to the analytical method described by Tsakona et al. [3]. The lipid composition was determined using a gas chromatograph (Fisons 8060 unit) equipped with FID detector and using a Chrompack column (60 m × 0.25 mm, film thickness 0.25 mm, J&W Scientific). Helium was used as carrier gas, at a flow rate of 2 mL/min. Oven temperature was started from 200°C and held for 13 min, increased to 220°C at a rate of 2°C/min and then with a rate of 20°C/min at 240°C and held for 3 min. The injector and the detector temperature were adjusted at 250°C.

2.8 Calculation of biodiesel properties

The properties of FAME namely length of chain (LC), cetane number (CN), low calorific value (LCV), cold filter plugging point (CFPP), flash point (FP) and kinematic viscosity (μ) were estimated based on the equations of Pinzi et al. [12] and Ramos et al. [7] (Table 1).

3 Results and discussion

3.1 Isolation and screening

Several species such as *Rhodosporidium* sp., *Rhodotorula* sp., *Yarrowia sp.*, *Cryptococcus* sp., *Lipomyces* sp. are well known for intracellular lipid accumulation when cultivated on various carbon sources. Recent studies focus on the isolation of newly oleaginous yeasts from different environments (e.g. soil, plant materials, waste of palm oil production processes and biodiesel plants, flowers, glacial environments) with the ability to accumulate microbial oil under nutrient limiting conditions [13–16]. In this study, a total of 88 yeast strains were isolated from nine species of fruits. Yeast isolates were cultivated in shake flasks with sucrose as carbon source in nitrogen-limited media to direct the microbial metabolism towards the synthesis of secondary metabolites. Fifty two strains which showed lipid bodies inside the cells were selected and evaluated for their ability to accumulate lipids. The total lipid content of twenty one isolates was more than 20% (w/w) of the total dry cell weight indicating their oleaginous nature.

Among the lipid producing strains, the isolates PD_D2, VV_D4, PC_A2, PD_F1, PD_D3 and MD_F1 that showed high lipid content, were selected for further investigation. The total dry weight, lipid concentration and lipid content are shown in Table 2. The selected yeast strains showed high total dry weight

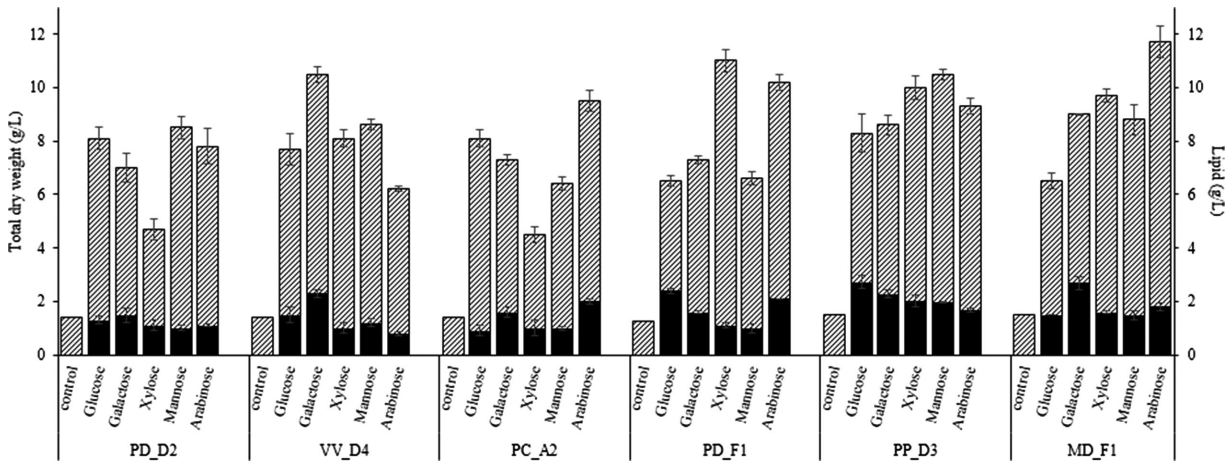

Figure 2. Total dry weight (grey bar) and lipid concentration (black bar) achieved by selected oleaginous yeasts using a range of carbon sources at an initial sucrose concentration of 20 g/L.

Table 4. Microbial oil production by oleaginous yeasts cultivated on various carbon sources in shake flask cultures

Carbon source	Strain	Initial sugar concentration (g/L)	Total dry weight (g/L)	Lipid (g/L)	Lipid content (%, w/w)	Lipid yield (g/g)	References
Glucose	*Rhodotorula glutinis* 2.107	50	4	0.5	13	0.05	[35]
	Lipomyces starkeyi 2.1390		6.2	2.3	37.2	0.11	
	Lipomyces starkeyi 2.1608		9.4	2	21.8	0.11	
	Rhodosporidium toruloides 2.1389		4.3	1.7	39.3	0.13	
	Rhodotorula glutinis 2.704		5.5	0.9	16.7	0.03	
	Rhodotorula glutinis	100	15.2	5.6	36.6	0.06	[14]
			29.8	14.7	49.3	0.15	
	Candida curvata[a)]	30	10.2	3.4	33.2	0.12	[22]
	Lipomyces starkeyi AS 2.1560	39	23.8	nr	53	0.18	[23]
	Rhodotorula glutinis IIP-30[a)]	30	5.1	0.4	7.5	0.03	[19]
	PD_D2	20	8.1 ± 0.40	1.3 ± 0.15	15.4 ± 1.09	0.06	This study
	VV_D4	20	7.7 ± 0.60	1.5 ± 0.30	19.3 ± 1.39	0.07	
	PC_A2	20	8.1 ± 0.30	0.9 ± 0.20	11.0 ± 1.06	0.04	
	PD_F1	20	6.5 ± 0.20	2.4 ± 0.10	37.0 ± 1.68	0.11	
	PP_D3	20	8.3 ± 0.70	2.7 ± 0.25	32.8 ± 1.75	0.12	
	MD_F1	20	6.5 ± 0.30	1.5 ± 0.01	23.1 ± 1.07	0.07	
Xylose	*Candida curvata*[a)]	30	9.9	4.8	48.6	0.17	[18]
	Lipomyces starkeyi AS 2.1560	70	22.3	12.7	57	0.18	[23]
	Lipomyces starkeyi AS2. 1390	nr	20.9	4.3	20.5	nr	[2]
	Rhodosporidium toruloides AS 2. 1389	nr	7.2	1.9	26.8	nr	
	Rhodotorula glutinis AS 2. 703	nr	6.9	0.8	12	nr	
	Rhodosporidium toruloides	40	12.5	4.9	38.9	0.15	[36]
	Lipomyces starkeyi	30	12.3	4.3	35	nr	[37]
	PD_D2	20	4.7 ± 0.40	1.1 ± 0.20	23.2 ± 1.28	0.09	This study
	VV_D4	20	8.1 ± 0.30	1.0 ± 0.20	12.5 ± 1.93	0.06	
	PC_A2	20	4.5 ± 0.30	1.0 ± 0.30	22.8 ± 0.18	0.13	
	PD_F1	20	11 ± 0.40	1.1 ± 0.10	10.0 ± 0.55	0.06	
	PP_D3	20	10 ± 0.43	2.0 ± 0.21	19.9 ± 1.26	0.1	
	MD_F1	20	9.7 ± 0.25	1.6 ± 0.05	16.6 ± 0.95	0.08	
Galactose	*Trichosporon fermentans*	100	23.6	13.9	59	0.15	[38]
	PD_D2	20	7.0 ± 0.55	1.5 ± 0.27	21.4 ± 1.19	0.11	This study
	VV_D4	20	10.5 ± 0.30	2.3 ± 0.15	21.5 ± 1.04	0.1	
	PC_A2	20	7.3 ± 0.18	1.6 ± 0.19	21.8 ± 1.11	0.13	
	PD_F1	20	7.3 ± 0.13	1.6 ± 0.06	21.9 ± 0.51	0.13	
	PP_D3	20	8.6 ± 0.35	2.3 ± 0.15	27.3 ± 0.58	0.15	
	MD_F1	20	9.0 ± 0.03	2.7 ± 0.25	29.4 ± 1.85	0.13	
Mannose	*Trichosporon fermentans*	100	22.7	11.5	50.4	0.14	[38]
	PD_D2	20	8.5 ± 0.40	1.0 ± 0.10	11.7 ± 0.62	0.05	This study
	VV_D4	20	8.6 ± 0.20	1.2 ± 0.14	14.4 ± 1.35	0.06	
	PC_A2	20	6.4 ± 0.25	1.0 ± 0.08	15.0 ± 0.67	0.05	
	PD_F1	20	6.5 ± 0.24	1.0 ± 0.17	14.4 ± 1.08	0.05	
	PP_D3	20	9.5 ± 0.20	2.0 ± 0.01	21.0 ± 0.34	0.1	
	MD_F1	20	8.8 ± 0.55	1.5 ± 0.17	16.8 ± 0.94	0.06	
Arabinose	*Lipomyces starkeyi* AS2. 1390	nr	14	3.5	24.9	nr	[2]
	Rhodosporidium toruloides AS 2. 1389	nr	4.8	0.8	16.8	nr	
	Rhodotorula glutinis AS 2. 703	nr	4.3	0.2	4.9	nr	
	PD_D2	20	7.8 ± 0.65	1.1 ± 0.05	14.2 ± 0.55	0.07	This study
	VV_D4	20	6.2 ± 0.10	0.8 ± 0.08	13.5 ± 1.07	0.07	
	PC_A2	20	9.5 ± 0.40	2.0 ± 0.09	21.1 ± 0.08	0.09	
	PD_F1	20	10.2 ± 0.30	2.0 ± 0.01	20.6 ± 0.49	0.1	
	PP_D3	20	9.3 ± 0.30	1.7 ± 0.06	17.8 ± 1.27	0.08	
	MD_F1	20	11.7 ± 0.60	1.8 ± 0.15	15.7 ± 0.48	0.08	

nr: not reported
[a)]batch bioreactor

(5.6–8.4 g/L), while the lipid content was in the range of 25–48.6 % (w/w).

The utilization of sucrose as a carbon source for microbial oil production has been reported in a few studies. *Candida curvata* and *Rhodotorula glutinis* have been studied for microbial oil production using sucrose as a carbon source, however only *Candida curvata* could accumulate high lipid contents (34%, w/w) in batch cultures [17–19]. Several yeast strains (e.g. *Rhodotula* 110, *Cryptococcus podzolicus*, *Trichosporon porosum*, *Pichia segobiensis*, *Trichosporonoides spathulata*, *Kodamaea ohmeri*, *Cryptococcus* sp. and *Cryptococcus musci*) have been isolated and studied for microbial oil production using various carbon sources as well as agricultural residues [15, 16, 20]. Promising results have been reported by Enshaeieh et al. [20], and Kitcha and Cheirsilp [15] using *Rhodotula* 110 and *Trichosporonoides spathulata*, respectively. *Rhodotula* 110, isolated from soil, exhibited the highest lipid content (58.2%, w/w) in optimized conditions with glucose as carbon source, while the use of lignocellulosic material (corn stalk and wheat straw hydrolysate) was also studied and the results indicated high potential for lipid accumulation (38.9 and 43.4%, respectively) [15, 20]. Moreover, the isolated oleaginous yeast *Trichosporonoides spathulata* could produce lipids (42.8%, w/w) using crude glycerol as carbon source [15].

3.2 Identification of oleaginous yeast strains

The six yeast isolates that were selected for further investigation were identified to their closest known relatives based on the partial 26S-rRNA gene sequence. Figure 1 represents the phylogenetic relationship of six newly isolated yeasts with oleaginous and non-oleaginous yeast type strains and Table 3 represents the phylogenetic position of the six isolates based on the D1/D2 region of 26S-rRNA gene sequence. All strains appeared to belong to the genus *Metschnikowia* and the identity percentage to their closest relatives was 97–99%. Species that belong to the genus *Metschnikowia* have been mainly studied for ethanol production during the first stages of alcoholic fermentation in wine making [9]. There has been one report for the production of microbial oil by *Metschnikowia pulcherrima* [21]. Open air stirred tank reactors of 500 L were used for the cultivation of *Metschnikowia pulcherrima* in a temperature controlled glasshouse, under non-sterile conditions, at low pH using glycerol as a carbon source. The total dry weight after 15 days was around 2 g/L with a lipid content of 34% (w/w).

3.3 Microbial oil production from different carbon sources

The ability of the selected isolates to accumulate lipids on a wide range of carbon sources, including glucose, xylose, galactose, mannose and arabinose, was evaluated. Figure 2 presents the total dry weight and lipid concentration achieved by the selected strains in media with initial carbon source of 20 g/L when maximum lipid accumulation was achieved. All strains have the ability to consume all C5 and C6 sugars and accumulate microbial oil. The consumption of different carbon sources led to varying lipid accumulation and fatty acid composition. Xylose consumption led to the highest total dry weight for isolates PD_F1 and PP_D3. In the case of PC_A2 and MD_F1, the total dry weight reached the highest values when arabinose was used as a carbon source, while isolates PD_D2 and VV_D4 presented the highest total dry weight production in mannose and galactose, respectively. The utilization of glucose resulted in high lipid accumulation for isolates PD_F1 and PP_D3, where the lipid concentrations achieved were 2.4 g/L and 2.7 g/L corresponding to intracellular lipid contents of 37% (w/w) and 32.8% (w/w), respectively. Galactose consumption led to the highest intracellular lipid contents for strains PP_D3 (27.3%) and MD_F1 (29.4%). Furthermore, PD_D2 and PC_A2 have shown higher lipid content in xylose, despite the low total dry weight produced. Among the carbon sources tested, arabinose, glucose, galactose and xylose resulted in the highest lipid concentration for PD_F1, PP_D3, MD_F1 and PP_D3, respectively. The consumption of mannose led to relatively high lipid accumulation (21%, w/w) only in the case of PP_D3. Comparing the isolates, PP_D3 reached the maximum lipid concentration in all substrates ranging from 2 to 2.7 g/L, except for arabinose.

Table 4 presents lipid production reported in various literature-cited publications using different oleaginous yeasts cultivated mainly in shake flasks on C5 and C6 sugars. Although glucose has been extensively studied as carbon source for microbial oil production, many oleaginous yeasts show high efficiency on lipid production using xylose as carbon source. *Lipomyces starkeyi* and *Candida curvata* have been reported to accumulate lipids up to around 50% of their total dry weight [22, 23]. Even though arabinose contributes 1 to 18% of the total carbohydrates in hydrolysates there are limited publications reporting lipid production from arabinose, where one of the highest lipid contents (25%, w/w) was achieved by *Lipomyces starkeyi* [2].

3.4 Fed-batch fermentation using hydrolysates from flour-rich waste streams

Following the promising results of the screening study, a waste stream from the confectionary industry was used for the

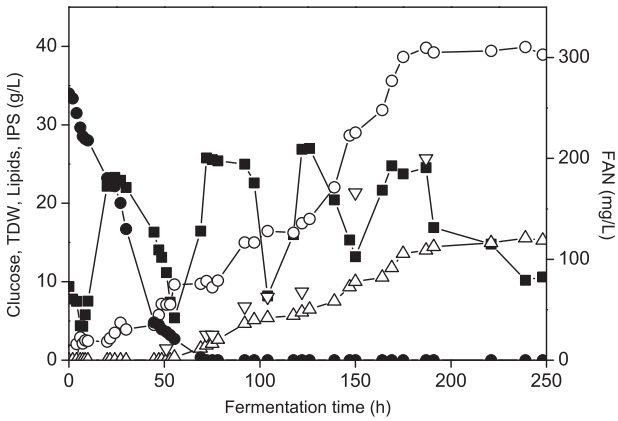

Figure 3. Consumption of glucose (■), FAN (●) and production of TDW (○) and lipids (△) during fed-batch fermentation of VV_D4 using FRW hydrolysate.

Table 5. Fatty acid composition (%) of microbial oils produced by the selected strains on various carbon sources (shake flask fermentations have been carried out in triplicate) and range of fatty acid composition (%) of common vegetable oils and animal fats

Strain	Substrate	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Others
PD_D2	Glucose	12.5	7.3	3.9	54.2	15.5	1.6	5.1
	Galactose	19.0	4.9	24.9	36.0	13.7	0.0	1.5
	Xylose	16.9	8.6	2.7	53.5	11.1	1.8	5.2
	Mannose	12.3	7.0	0.0	58.8	17.8	0.0	4.1
	Arabinose	15.2	6.0	28.0	44.9	0.0	1.5	4.4
VV_D4	Glucose	13.9	7.9	9.6	53.4	13.1	1.5	0.6
	Galactose	14.1	9.5	10.4	51.9	11.4	1.0	1.6
	Xylose	15.6	8.6	2.8	53.8	13.8	1.4	3.9
	Mannose	13.4	7.1	0.0	62.7	14.2	0.0	2.7
	Arabinose	16.9	9.8	2.9	61.2	9.2	0.0	0.0
PC_A2	Glucose	13.6	8.4	1.4	58.3	14.1	0.3	3.9
	Galactose	15.7	8.1	7.6	52.1	12.9	1.3	2.3
	Xylose	24.3	2.1	9.9	44.7	8.6	1.2	9.2
	Mannose	13.2	7.7	0.0	59.6	19.5	0.0	0.0
	Arabinose	19.7	7.9	17.9	48.9	5.3	0.0	0.3
PD_F1	Glucose	13.8	2.4	54.9	21.1	7.9	0.0	0.0
	Galactose	26.1	3.5	6.7	52.0	11.6	0.0	0.0
	Xylose	17.6	6.3	3.0	54.5	13.1	1.2	4.3
	Mannose	15.8	5.6	0.0	60.5	18.1	0.0	0.0
	Arabinose	16.3	6.0	10.9	55.3	9.9	1.0	0.5
PP_D3	Glucose	16.5	1.8	41.7	28.3	11.7	0.0	0.0
	Galactose	18.5	7.0	6.6	53.1	11.1	1.0	2.8
	Xylose	20.0	7.8	7.8	48.8	11.1	1.3	3.2
	Mannose	18.0	5.9	3.2	59.5	11.5	0.0	1.7
	Arabinose	15.4	6.8	9.0	55.5	11.4	1.2	0.7
MD_F1	Glucose	14.3	7.4	3.1	57.6	14.6	0.0	3.0
	Galactose	14.9	6.0	10.9	55.3	9.9	1.0	1.9
	Xylose	16.4	6.8	9.0	51.2	11.5	1.2	3.8
	Mannose	18.0	5.9	3.2	59.5	11.5	0.0	1.7
	Arabinose	17.2	0.0	0.0	60.1	22.8	0.0	0.0
VV_D4	RFW	28.8	0.0	15.9	41.6	13.7	0.0	0.0

Oils and fats	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	References
Sunflower oil	5–7	–	3-6	14–40	48–74	–	[28]
Soybean oil	10–12	–	3–5	18–26	49–57	6–9	
Cottonseed oil	22–26	–	2–3	15–22	47–58	traces	
Rapeseed oil	2–5	–	0–4	51–68	18-25	7–11	[39]
Palm oil	39–50	–	3–5	38–45	8–12	traces	
Tallow	24–32	–	20–25	37–43	2–3	–	[40]
Lard	28–30	–	12–18	40–50	7–13	0–1	
Yellow grease	23.24	–	12.96	44.32	6.97	0.67	
Brown grease	22.83	–	12.54	42.36	12.09	0.82	

production of fermentation media in order to evaluate the potential for microbial oil production by the isolate VV_D4. Specifically, flour-rich waste hydrolysate was used as carbon source. An important parameter that influences the microbial oil accumulation in oleaginous yeasts is the nitrogen content of the respective cultivation media. A nutrient complete medium was used at the beginning of the fermentation in order to achieve a high cell density. The initial FAN concentration was 260 mg/L. The accumulation of lipids was achieved via continuous supply of a glucose-rich feeding solution when nitrogen limitation was attained. Figure 3 presents the glucose, FAN, TDW, and lipid concentration obtained during cultivation of VV_D4 on FRW hydrolysate. The TDW reached was 40 g/L with an intracellular lipid content of 39% (w/w) after 240 h. Lipid accumulation started when FAN was depleted from the fermentation broth. The accumulation of endo-polysaccharides was also observed after FAN was depleted along with lipid production. From 50 to 120 h endo-polysaccharide formation was observed to increase gradually from 0.21 to 0.5 g-IPS/g-TDW. Until the end of the fermentation, the IPS content remained stable at 0.5 g-IPS/g-TDW.

The valorization of waste from the food industry has been rarely used as raw material for microbial oil production by oleaginous yeasts. Tsakona et al. [3] has reported the utilization of flour rich hydrolysate for microbial oil production by *Lipomyces starkeyi* reaching a TDW of 109.8 g/L with lipid content of 57.8% (w/w) in fed-batch fermentation. Moreover, fish wastewater supplemented with glucose has been used by

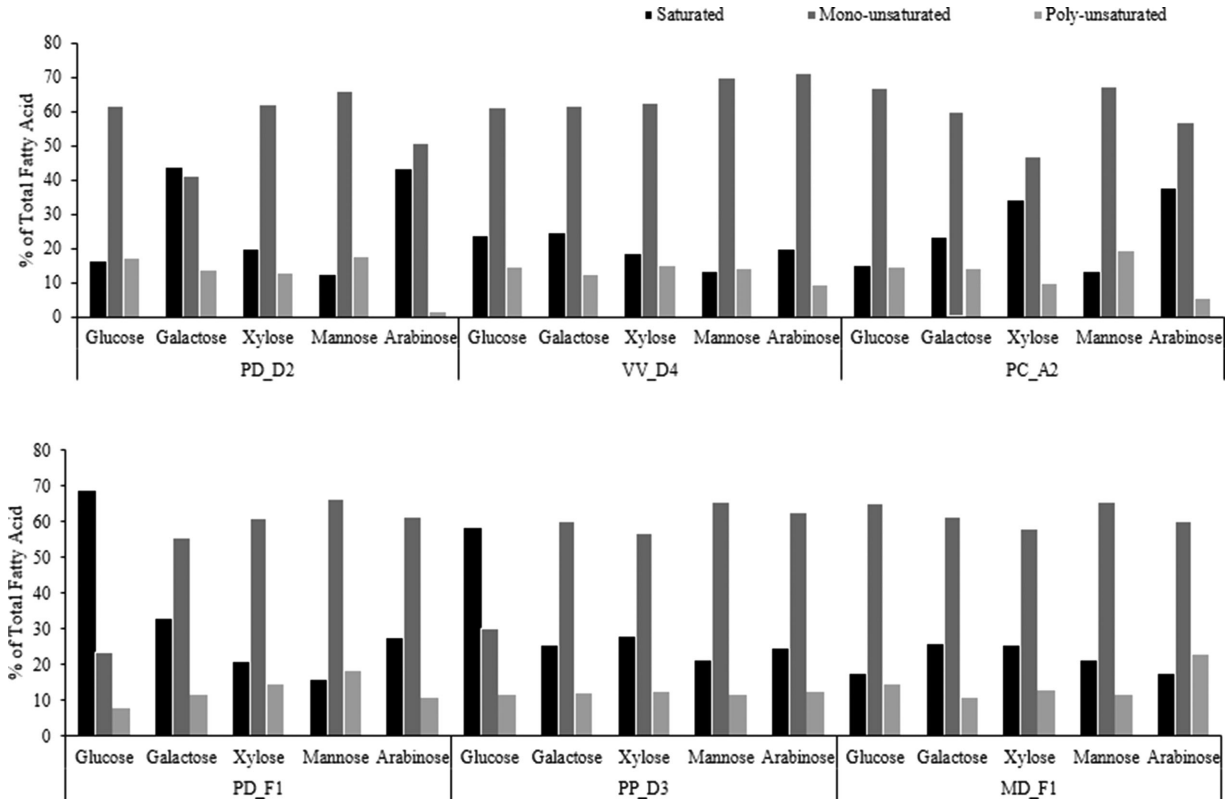

Figure 4. Effect of carbon source on saturated, mono-unsaturated and poly-unsaturated fatty acid composition.

Lipomyces starkeyi in shake flask culture where the highest lipid concentration achieved was 2.7 g/L [24].

3.5 Lipid composition

The fatty acid composition of microbial oil is significantly affected by the microorganism, the substrate and the culture conditions employed. In most oleaginous microorganisms, the fatty acid profile of microbial oil contains mainly C16 and C18 fatty acids. Based on the fatty acid profile, microbial oils could have various applications for the production of biodiesel, surfactants, waxes, lubricants and chemical feedstocks [6, 25]. In this study, the fatty acid composition of lipids produced in the different carbon sources was determined in order to evaluate the potential use of lipids as raw material for biodiesel production. Table 5 presents the fatty acid content produced by the selected yeast isolates when cultivated on different carbon sources when maximum lipid accumulation was achieved. Table 5 also presents the fatty acid profile of common vegetable oils and animal fats. The produced lipids mainly contain fatty acids with 16 and 18 carbon atoms. Specifically, the predominant fatty acids were oleic acid (18:1) followed by mainly palmitic acid (C16:0), stearic acid (18:0) and linoleic acid (18:2). The fatty acid profiles are similar to the common vegetable oil feedstocks used for biodiesel production. In particular, a total content of saturated fatty acids of about 68.7% was produced by PD_F1 when cultivated on glucose, whereas the highest content of oleic acid (62.7%) was observed by VV_D4 using mannose as substrate (Fig. 4). The lipids that contain more than 50% (w/w) of mono-unsaturated fatty acids have similar fatty acid composition to rapeseed oil (Table 5). The microbial lipids that contain more than 50% (w/w) of saturated fatty acids have similar fatty acid composition to most animal fats and in some cases palm oil. The fatty acid profile of the microbial oil produced by isolate VV_D4 in fed-batch fermentation was similar to most animal fats.

In some cases, the fatty acid composition was significantly affected by the type of carbon source provided and also by the yeast strain used. For instance, the fatty acid profiles produced by PD_D2 in arabinose and galactose were similar to tallow, while the microbial oil produced by PP_D3 and PC_A2 on xylose and PD_F1 on galactose primarily consisted of palmitic and oleic acids. Moreover, when the isolates PD_F1 and PP_D3 were cultivated on glucose, a high content of saturated fatty acid (13.8% and 16.5% of palmitic acid and 54.9% and 41.7% of stearic acid, respectively) was produced. Similar compositions of fatty acids have been reported by Easterling et al. [26] with *Rhodotorula glutinis* cultivated on xylose and glycerol, where the fraction of saturated FAMEs were 69 and 68%, respectively [26]. Furthermore, the fatty acid composition of the lipids produced by *Candida tropicalis* using molasses as carbon source contained a high content of palmitic acid (29.7%) and stearic acid (56.2%) [27].

Table 6. Biodiesel properties based on the fatty acid composition of the microbial oil produced by selective isolates in different carbon sources

Strain	Substrate	CN	UD	LC	LCV (kJ/kg)	FP (°C)	M (mm^2/s)	CFPP (°C)
PD_D2	Glucose	53	0.97	16.69	37133.18	143.76	4.69	−2.28
	Galactose	61	0.68	17.25	37519.28	167.27	5.48	9.4
	Xylose	54	0.9	16.56	37097.08	141.75	4.71	−0.62
	Mannose	53	1.01	16.88	37211.4	148.04	4.75	−4.75
	Arabinose	62	0.55	16.78	37342.25	155.74	5.38	10.48
VV_D4	Glucose	57	0.92	17.45	37525.1	167.43	5.25	−0.96
	Galactose	57	0.87	17.24	37438.54	161.59	5.18	−0.01
	Xylose	54	0.94	16.81	37202.65	147.41	4.81	−1.82
	Mannose	55	0.98	17.11	37336.6	155.22	4.94	−4.93
	Arabinose	58	0.89	17.47	37542.34	168.63	5.29	−3.08
PC_A2	Glucose	54	0.96	16.86	37222.97	148.57	4.82	−3.56
	Galactose	56	0.9	17.1	37364.36	156.85	5.05	−0.26
	Xylose	56	0.68	15.81	36821.66	128.9	4.56	8.43
	Mannose	55	1.06	17.58	37533.73	168.08	5.12	−6.32
	Arabinose	62	0.67	17.39	37592.59	172.55	5.59	5.75
PD_F1	Glucose	68	0.39	17.68	37839.33	193.33	6.29	20.75
	Galactose	60	0.79	17.41	37555.17	169.63	5.42	3.27
	Xylose	55	0.91	16.74	37184.99	146.39	4.82	−0.6
	Mannose	56	1.02	17.57	37544.33	168.78	5.17	−5.04
	Arabinose	59	0.84	17.46	37560.85	169.97	5.37	0.8
PP_D3	Glucose	66	0.53	17.63	37763.6	186.27	6	15.68
	Galactose	57	0.85	16.99	37326.92	154.53	5.05	0.86
	Xylose	57	0.83	16.87	37278.75	151.63	5.01	2.39
	Mannose	57	0.89	17.21	37419.41	160.35	5.14	−1.53
	Arabinose	58	0.89	17.43	37528.32	167.66	5.28	−0.5
MD_F1	Glucose	55	0.94	17.03	37311.13	153.64	4.95	−2.79
	Galactose	58	0.84	17.24	37453.6	162.59	5.23	0.8
	Xylose	56	0.85	16.86	37265.2	150.85	4.97	1.51
	Mannose	57	0.89	17.21	37419.47	160.35	5.14	−1.53
	Arabinose	56	1.06	17.66	37572.3	170.76	5.17	−4.36
VV_D4	FRW	62	0.69	17.42	37600.73	173.11	5.59	9.08
EN 14214		>51				>120	3.5-5	
ASTM D6751		>47				>93	1.9-6	

3.6 Biodiesel properties

Fuel properties depend on the chemical structure of the fatty acid methyl esters. Predictive equations and mathematical models have been developed and used to predict some parameters in the biodiesel based on the FAME composition [7]. In this study, the different physicochemical biodiesel properties were determined using mathematical equations based on the FAME profile and compared with the international standards. Table 6 presents the properties of oil produced by yeast isolates cultivated on different carbon sources and also the USA and European specifications regarding the accepted biodiesel properties.

In general, saturated long chain fatty acids excel in oxidation stability, cetane number and flash point, while exhibit poor cold flow properties, higher kinematic viscosity and lower calorific value [28]. The biodiesel generated from microbial oils produced by the isolates cultivated in all carbon sources are within the limits set by EN 14214 and ASTM D6751 regarding cetane number and flash point. Cetane number is a primary indicator of fuel quality which is related to the ignition delay time. The predicted cetane number of microbial oil produced by the isolates were in all cases high, due to the high content of methyl esters of saturated fatty acids (stearic and palmitic acids). Regarding flash point the values of the microbial oil produced by the yeast strains are more than 128°C making the biodiesel safe to handle, store and transport.

Significant differences in terms of CFPP and kinematic viscosity were found for yeast oil derived biodiesel. CFPP is an important parameter that describes the fluidity in low temperature and depends on the regulations of each country. Saturated and long chain fatty acids have an adverse effect on cold flow properties of biodiesel. Oils with high saturated degree have higher melting points causing crystallization of the esters in low temperatures [29].

Several methods have been employed to improve the cold flow properties of biodiesel. Blending biodiesel with petroleum diesel or use of cold flow improvers could result in better value regarding cold flow properties [30]. Winterization is another method to improve cold flow properties by removing saturated methyl ester [31]. Wang et al. [32] investigated the addition of surfactants for reduction of CFPP where the addition of polyglycerol ester has the greatest effect to the CFPP [32].

Concerning the kinematic viscosity calculated by the predictive model showed that only some microbial oils fulfill the limit set by EN 14214, while in the case of the limit set by ASTM D6751 the vast majority of microbial oils lead to acceptable values of kinematic viscosity. High kinematic viscosity could affect the atomization of fuel on injection which has negative effects on the performance of the engine. Several techniques could be employed in order to reduce the kinematic viscosity such as preheating the oils, blending or dilution with other fuels in appropriate ratios, thermal cracking / pyrolysis, ultrasonic treatment and supercritical methanol trans-esterification [33, 34].

4 Concluding remarks

Transition to the bio-economy era from the petroleum-based economy is a challenging process while the demand for renewable feedstocks has increased. Industrial waste and by-product streams constitute valuable renewable resources for the production of commodity chemicals, bio-polymers and fuels. The six new isolates identified in this study are promising for the production of microbial oil and biodiesel using various carbon sources. Waste streams from the confectionary industry have been used in this study for the production of 15 g/L microbial oil via fed-batch bioreactor cultures.

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The authors declare no conflict of interest.

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