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Microbial Biotechnologies to Produce Biodiesel and Biolubricants from Dairy Effluents

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Abstract: The shift from fossil fuels to renewable energy sources is crucial in addressing environmental challenges. Vegetable oils have been focused on as the main potential source for biodiesel and biolubricant production. However, due to their fatty acid (FA) composition they are characterized by low stability to oxidation and variable viscosity. Single-cell oils (SCOs) from oleaginous microorganisms are a possible alternative to vegetable oils: their composition is more suitable, and it can further be improved by controlling the fermentation's physiological conditions. In the present study, the production of SCOs with targeted technological properties from *Lipomyces starkeyi* in fermentation under controlled temperatures was assessed. A dairy effluent (scotta) was used as the fermentation substrate to improve the economic sustainability of the process. Batch aerobic fermentations were carried out in a fermenter at two different temperatures (25 °C and 30 °C). The fermentation yields and SCO FA profiles were analyzed. The highest yields of biomass (9.76 g L^{-1}) and microbial oil (1.83 g L^{-1}) were obtained from fermentations carried out at 30 °C. Furthermore, a significantly lower content (46% vs. 55%) of unsaturated FAs and higher content (11% vs. 1.5%) of shorter-chain saturated FAs, with myristic acid almost matching stearic acid, were detected at 30 °C in comparison to 25 °C. Very low peroxide values were also found (0.14 meq O_2 kg^{-1} at 30 °C and 0 meq O_2 kg^{-1} at 25 °C). These results indicate that these SCOs were highly oxidation-resistant, and that a higher fermentation temperature improves their oxidative stability and tribophysical features. The biodiesels' technological properties, calculated from the FA composition, were within the limits of both U.S. standards and E.U. regulations. Then, SCOs produced from *L. starkeyi* by fermentation of dairy effluents carried out under controlled temperature can be considered a suitable alternative to vegetable oils to produce biodiesel and biolubricants.

Keywords: *Lipomyces starkeyi*; scotta; single-cell oil; oleaginous yeasts; biorefineries; circular economy

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1. Introduction

Replacing fossil fuels with renewable sources to limit the environmental concerns associated with the use of non-renewable energy sources [1] is a complex challenge, and bio-based products, biofuels, and oleochemicals have become an important alternative in recent years [2]. The global supply of biodiesel in 2021 was 0.8 million barrels per day (mb/d), and a further increase is expected in the upcoming years, reaching 1.8 mb/d by 2045 [3]. Indonesia is the world's largest biodiesel producer: in 2022, Indonesia had a reported output of 13.65 billion liters, more than the combined amount produced by Brazil and the United States, which are the second- and third-largest producers, respectively [4]. Edible feedstocks such as wheat, rice, potatoes, sugar cane, barley, and vegetable oil are used to produce first-generation biofuels, and vegetable oils are the main renewable sources

used for biodiesel and biolubricant production [5]; however, a shift towards the use of non-edible biomasses is ongoing, in order to avoid the competition with food production and to reduce the extensive land consumption under a worldwide rising demand for biodiesel [2,6]. Recently, some reports documented the use of bioenergy crops for co-production of oils and sugars for biodiesel and bioethanol production [7]. Also, microbial oils, called "single-cell oils" (SCOs), are emerging as a promising alternative to vegetable oils for the production of biofuels, biodiesel, oleochemicals, biolubricants, soap, plastics, and other bio-products [8–15]. SCOs can be produced in fermentation plants, without land consumption and without dependence on weather seasonality, and wastes and effluents can be used as substrates for fermentation, with a significant role in a circular economy approach. SCOs can also compete with vegetable oils from a qualitative point of view; indeed, vegetable oils have several significant drawbacks for technological features when compared to fossil fuels, such as poor stability to hydrolytic, thermal, oxidative and biological degradation, and poor atomization due to high viscosity [6,16,17]. Vegetable oils' low oxidative stability is due to a high content of unsaturated fatty acids, that, following a radical attack, give rise to insoluble deposits and increase the viscosity. The production cost of SCOs is higher than that of vegetable oils, even if the SCOs produced from yeast biomasses can be considered economically suitable for specific uses [18,19]. However, a rising trend in the costs of vegetable oils has been observed over recent years due to the rising demand for biodiesel production; moreover, the economic sustainability of the SCO processes can be strongly improved when negative-cost raw materials, such as wastes or effluents, are used as fermentation media, and a further cost reduction can be reached by the direct transesterification of the biomass, thus avoiding the oil extraction step, which is one of the most costly steps of the SCO production procedure [19–21].

Microbial lipids are synthesized in cells with different functions, such as membrane components, storage lipids, or for regulatory functions [22]. Several oleaginous microorganisms, storing high amounts of lipids (>20% *w*/*w*) in their cells, can be used to produce SCOs [23]. Oleaginous yeasts, such as *Rhodotorula glutinis*, *Rhodotorula graminis*, *Rhodosporidium toruloides*, *Lipomyces starkeyi*, *Yarrowia lipolytica*, *Cryptococcus albidus*, and *Cutaneotrichosporon oleaginosus*, can convert carbon sources into storage-neutral lipids, mainly triacylglycerols, reaching lipid contents of more than 65% of their dry cell weight [24–26]. The amount of lipids stored in yeast cells is influenced by several factors such as cultivation method (batch, fed-batch, or continuous), growth medium composition, C/N ratio, carbon source provided, temperature, and air availability [25,27,28]. Their fatty acid profile is very similar to that of vegetable oils, which they can replace for several uses [25,29,30], but in general the unsaturated fatty acid content is lower, and thus, their oxidative stability is higher. The most abundant fatty acids produced by oleaginous yeasts are C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid) and C18:2 (linoleic acid), whereas the fatty acids such as C14:0 (myristic acid) and C18:3 (linolenic acid) are less abundant [23]. Many studies have shown that yeast SCOs are suitable to produce biodiesel and biolubricants, and that their tribo-chemical properties, such as lubricity, viscosity index, emissions production, biodegradability, and no dermatological problems for humans, are better than vegetable oils [11,27,31,32].

Different yeast species and strains can grow on different carbon sources (glucose, xylose, arabinose, mannose, glycerol, lactose, galactose) and on agricultural and industrial residues; therefore, yeasts are suitable for SCO production as low-cost carbon sources can be easily used [23,27,33]. Several industrial by-products or wastes have been tested as substrates, such as molasses, dairy effluents, pulp and paper mill effluent, sewage sludge, and hydrolysate from different biomasses, such as wheat straw, corn stover, rice straw, sugarcane bagasse, coniferous and deciduous woods, and grasses [34–36]. Among them, effluents from the dairy industry play a major role. The processing of 10 kg of milk produces approximately 1–2 kg of dairy products and 8–9 kg of effluents [37]; 187–206 million tonnes of dairy effluents are estimated to be produced yearly around the world, with 40 million

tonnes produced within the E.U. alone [38]; their pollution load is significant, due to the high COD and BOD [39].

Dairy effluents include cheese whey and scotta [40]. Over the years, research has focused mainly on the recycling of cheese whey, while less attention has been paid to the recovery of scotta, also known as ricotta cheese exhausted whey (RCEW); the latter is the liquid remaining after the production of ricotta cheese, which is obtained from cheese whey by coagulating proteins through the combined action of high temperature and acidity [41]. Scotta is mainly produced in Italy and other Mediterranean countries. Italian production is estimated to be around 1.0 million tonnes per year, leading to significant environmental concerns regarding its disposal [42]. Scotta's main component is lactose (3.7–5.0%), followed by salts (1–1.13%), proteins (0.15–0.22%), and fats (0.1–0.3%). Its BOD (biochemical oxygen demand) and COD (chemical oxygen demand) values are high, 50 g L^{-1} and 80 g L^{-1}, respectively [43]; therefore, it requires appropriate treatment to avoid environmental concerns.

Dairy effluents can find use in microbial biotechnological processes as growth media for fermentations due to their high sugar content. In particular, scotta can be considered suitable as a growth medium for SCO production due to its high C/N ratio; moreover, several yeasts strains can use lactose as a carbon and energy source.

In this work, the oleaginous yeast *Lipomyces starkeyi* was used to produce SCOs using scotta as the substrate. The main aim was to create SCOs with compositions suitable for the production of biodiesel and biolubricants by controlling the physiological conditions during fermentation; indeed, different growth temperatures allow for modification of the lipids composition in yeast, varying the balance between saturated and unsaturated fatty acids and between medium-chain and long-chain saturated fatty acids [25]. By adjusting the fatty acid profile, it is possible to improve the technological properties of the oil, in order to obtain a suitable fluidity together with a better oxidative stability, reached by reducing the unsaturation degree. So, batch fermentations were carried out under controlled conditions at two different growth temperatures, consistent with yeast viability and biomass yield, but also potentially effective for reaching different cell lipid compositions: 25 °C and 30 °C. A temperature of 28 °C is considered the best temperature to combine biomass and lipid yields in *Lipomyces starkeyi*, but several fermentations carried out at 30 °C and at 25–26 °C have also been reported [28]; our aim was to evaluate if the fatty acid profile reached at 25 °C could be more suitable for biodiesel and biolubricants production in comparison to that obtained at 30 °C. Temperatures lower than 25 °C were excluded due to the known significant prevalence of polyunsaturated fatty acids [44]. The oils' fatty acid profiles were analyzed, and fermentation yields were checked as valuable data for possible industrial process suitability. Technological properties were detected based on the fatty acid composition, and oxidative stability was analyzed. The residual sugars at the end of the fermentations were also examined to evaluate the beneficial effect of fermentation in reducing the polluting load of scotta.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

Lipomyces starkeyi DSMZ 70,295 strain was used as inoculum. Stock cultures were maintained on YEPD agar plates (10 g L^{-1} yeast extract (Biolife, Milan, Italy), 20 g L^{-1} peptone (Biolife, Milan, Italy), and 20 g L^{-1} glucose (Sigma Aldrich, Milan, Italy) at 4 °C. Precultures were grown in aerated flasks in YEPD at 28 °C for three days.

2.2. Dairy Effluent and Its Chemical–Physical Characterization

Scotta was obtained from a dairy in Piedmont (Pianezza, Italy) immediately after the ricotta production, aliquoted, and stored at −25 °C. The mean pH was 5.7 ± 0.07, and the sugar content was 44 ± 4.8 g L^{-1} lactose, 0.45 ± 0.04 g L^{-1} galactose, and no glucose. For each fermentation, scotta was thawed at 4 °C and sterilized by Tyndallization (three times at 95 °C for 30 min). The pH was measured by a pH meter (CRISON BASIC 20, Barcelona,

Spain). Sugar concentrations were analyzed at the start and at the end of each fermentation by HPLC (Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a refractive index detector (RID), (RefractorMax 520, ERC, Tokyo, Japan), operating at 55 °C, and a Dionex UltiMate 3000 variable-wavelength detector (Thermo Fisher Scientific, Waltham, MA, USA) set at 210 nm, on a Metab-AAC BF series column (length = 300 mm; ID = 7.8 mm) (Isera, Düren, Germany); isocratic elution was carried out with 9 mM H_2SO_4 at a flow rate of 1 mL min^{-1} in an oven at 60 °C.

2.3. Fermentations

Batch fermentations were carried out under controlled conditions in a BIOSTAT A plus 1 L Sartorius bioreactor (Sartorius Stedim Biotech, Göttingen, Germany) at two different growth temperatures, 25 °C for treatments named LS25 and 30 °C for treatments named LS30; each treatment was executed in triplicate. Sterile scotta (800 mL) was used for each fermentation, added with 1 mL of sterile silicon antifoam solution (2% v/v) (Titolchimica S.p.A., Pontecchio Polesine, Italy) and 1×10^6 cells mL^{-1} starter inoculum. Sterile air was fluxed throughout the whole fermentation at 1 mL min^{-1} and the medium was stirred at 150 rpm. Dissolved oxygen and pH were monitored with an OXIFERM™ O_2 sensor (Hamilton, Bonaduz, Switzerland) and a pH electrode (Hamilton, Bonaduz, Switzerland), respectively. Cell growth was monitored daily by haemocytometer counting and intracellular lipid droplet accumulation was checked by microscopic analysis with a Leica DM2000 LED light microscope (Leica, Wetzlar, Germany). Fermentation was stopped when the stationary phase was reached: after 10 days at 30 °C and 7 days at 25 °C.

2.4. Lipid Extraction and Fermentation Yields

Cell biomass was collected by centrifugation at 6000 rpm (Centrifuge Eppendorf 5804 R) for 10 min at 20 °C, then washed with sterile water. The cells pellet was weighed, and then, total lipids were extracted according to Egger and Schuwduke [45], modified according to Belviso et al. [46], as follows: cell biomass was resuspended in 2 mL of boiling dimethyl sulfoxide (DMSO) and incubated at 100 °C for 1 h. Methanol (7.5 mL) and 25 mL methyl-tert-butyl ether (MTBE) were added and the mixture was shaken by vortex and incubated on a rotary shaker (VDRL STIRRER 711, ASAL S.r.L., Cernusco sul Naviglio, Italy) at 75 rpm for 1 h at room temperature, then stored overnight at 4 °C. On the second day, 6.2 mL of sterile water was added and after 10 min of incubation at room temperature the sample was centrifuged at 6000 rpm for 10 min at 4 °C. The upper (organic) phase was collected, and the lower phase was re-extracted with 10 mL of a MTBE:methanol:water mixture (100:30:25, $v/v/v$). The pooled upper phases were dried in a rotary evaporator (Laborota 4000-efficent, Heidolph Instruments, Schwabach, Germany) at 40 °C; vacuum 580 mmHg. The extracted oil was quantified through gravimetric analysis and the total weight expressed in grams. The oil was stored in 2.5 mL PP amber tubes under nitrogen at −20.0 °C until gas chromatographic analyses.

Fermentation yields were expressed in percentages and calculated as follows:
Yp/s, where p = oil produced (g) and s = sugars consumed (g);
Yp/x, where p = oil produced (g) and x = cell biomass (g);
Yx/s, where x = biomass produced (g) and s = sugars consumed (g).

2.5. Lipids Analysis and Characterization

The SCO's fatty acid composition was analyzed by gas chromatography. Methyl esters of fatty acids (FAMEs) were obtained by adding 2 mL heptane to approximately 0.1 g of SCO in a 5 mL screw-top tube; after shaking, 0.2 mL of 2 M methanol potassium hydroxide solution were added. The tube was closed thoroughly with a cap fitted with a PTFE joint and shaken vigorously for 30 s, then left to stand until phase separation. The upper solution was withdrawn and immediately used for gas chromatographic analysis [47].

The fatty acid composition was determined in accordance with COI/T.20/Doc. No. 33 [48]. A gas chromatography (GC) system (HRGC Mega 2 series 8560; Thermo Fisher

Scientific, Waltham, MA, USA) was used with a capillary column in fused silica (60 m; 0.32 mm ID; 0.2 µm film thickness) SP–2380 (Supelco, Bellefonte, PA, USA). The column temperature was programmed for a gradient increase from 70 to 165 °C at 20 °C per minute, 23 min at 165 °C, increase from 165 to 200 °C at 1.5 °C per minute, 5 min at 200 °C, then increase at 2 °C per minute from 200 to 220 °C, finally, held for 5 min. The detector temperature was 230 °C and hydrogen was used as the carrier gas at a column head pressure of 60 kPa. The samples (0.4 µL) were injected on-column. Peak dentification was tested with a mixture of standards supplied by Supelco (CRM18918 F.A.M.E. Mix, C8–C24, certified reference material, Supelco, Bellefonte, PA, USA) to provide the percent fatty acid composition.

The SCO's oxidative stability was analyzed by detecting the peroxide values spectrophotometrically by an automatized system (OxyTester ®, CDR s.r.l. Florence, Italy), based on the detection at 505 nm of a red complex of Fe^{3+} ions, produced by oxidation of Fe^{2+} from peroxides that form from unsaturated fatty acids in the presence of oxygen at high temperature [49]. Results are expressed as meq O_2 kg^{-1}.

The SCO's technical parameters were calculated from the FAME composition profile according to Cianchetta et al. [50]: iodine value (IV), saponification value (SV), degree of unsaturation (DU), long-chain saturation factor (LCSF), cold filter plugging point (CFPP), oxidative stability (OS), higher heating value (HHV), density (D), cetane number (CN), kinematic viscosity (KV), and percentage of linolenic acid (C18:3).

2.6. Statistical Analyses

ANOVA and least significant difference (LSD) analyses were carried out by assuming a *p*-value threshold $\leq$0.05. Data were processed using the Origin software (OriginPro, version 9.9, 2022).

3. Results

3.1. Yeast Cell Growth and Substrate Consumption

Yeast cell growth (Figures S1 and S2) was checked in order to identify the time needed to reach the stationary phase; it was 190/240 h at 30 °C and 120–168 h at 25 °C; therefore, the fermentations were stopped after 10 days at 30 °C, and after 7 days at 25 °C. The final mean cell concentration was 6.21 × 10^7 CFU mL^{-1} at 30 °C and 5.42 × 10^7 CFU mL^{-1} at 25 °C.

At the end of the fermentations, the pH of the medium had risen from an initial value of 5.7 ± 0.07 to 6.8 ± 0.98 in LS30 and to 6.2 ± 0.01 in LS25 (Figures S1 and S2).

The mean sugar content of sterile scotta was 44 ± 4.8 g L^{-1} lactose, 0.45 ± 0.04 g L^{-1} galactose, and no glucose. At the end of fermentation, the galactose was absent in LS25 and present only in traces (5 × 10^{-3} g L^{-1}) in LS30; residual lactose was higher in LS25 than in LS30; indeed, sugar consumption was significantly higher in LS30 than in LS25 ($p < 0.05$) (Table 1).

Table 1. Residual sugar concentration, percent of consumed sugars, and pH at the end of fermentation. LS30: fermentation carried out at 30 °C; LS25: fermentation carried out at 25 °C. Data are expressed as means of triplicate measurements. (*) indicates significant difference between treatments, (ns) indicates no significant difference following ANOVA ($p \leq 0.05$).

	Treatments		
	LS30	**LS25**	
Total residual sugars (g L^{-1})	17.73 ± 9.39	40.58 ± 1.63	*
Consumed sugars (%)	55.39 ± 24.05	16.86 ± 3.26	*
pH	6.8 ± 0.98	6.2 ± 0.01	ns

3.2. Fermentation Yields

The total cell biomass and SCOs produced are reported in Figure 1A,B. The experimental data show that higher oil production (1.25 g L^{-1}) was reached at 30 °C compared to 25 °C (0.83 g L^{-1}), even if the difference was not statistically significant. Also, the cell biomass production was higher at 30 °C (9.76 g L^{-1}) than at 25 °C (7.55 g L^{-1}). So, fermentations at 30 °C seemed to be more efficient for both oil and biomass production, even if the differences were not statistically significant. Figure 1C–E show the percent fermentation yields. Yp/s was 7.46% at 30 °C and 10.81% at 25 °C. Yx/s at 30 °C was 44% and 92% at 25 °C. Yp/x was 14.51% in LS30 and 11.28% in LS25. Statistical analysis did not reveal significant differences except for in Yx/s.

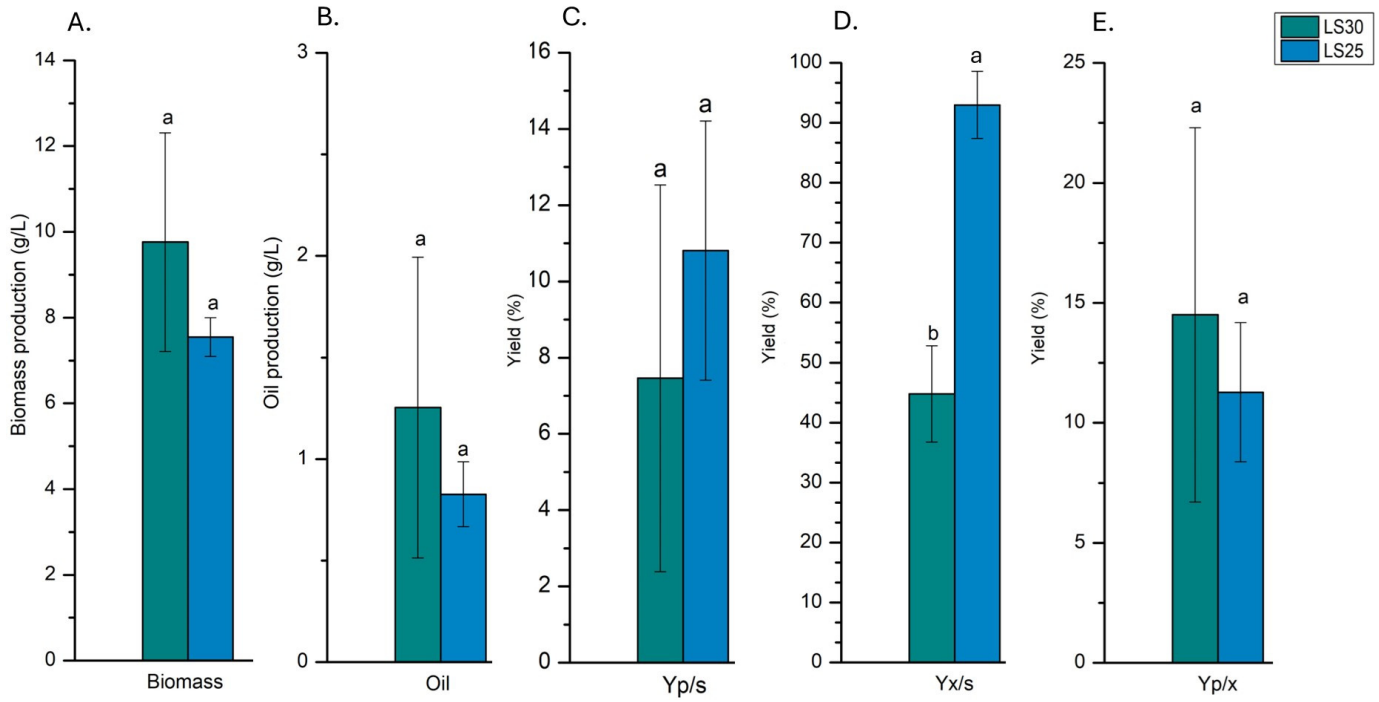

Figure 1. Fermentation yields. (**A**) Microbial cell biomass (g L^{-1}); (**B**) SCO (g L^{-1}). (**C**) Yp/s (%), where p = oil produced (g), and s = sugars consumed (g); (**D**) Yx/s (%), where x = biomass produced (g), and s = sugars consumed (g); (**E**) Yp/x (%), where p = oil produced (g), and x = cell biomass (g). LS30: fermentation carried out at 30 °C; LS25: fermentation carried out at 25 °C. Data are expressed as means of the triplicate measurements. Different letters show significant differences following ANOVA ($p \leq 0.05$).

3.3. Fatty Acid Profiles

The SCOs' FA profiles are shown in Table 2. The major FAs were oleic acid (C18:1) and palmitic acid (C16:0), with minor amounts of stearic (C18:0), myristic (C14:0), linoleic (C18:2), palmitoleic (C16:1), lauric (C12:0), capric (C10:0), and caprylic (C8:0) acids. Several odd-chain FAs were also detected. Statistical analysis revealed significant differences in the fatty acid profiles due to fermentation temperature. Among the medium-chain saturated FAs, significant differences were found for caprylic, capric, undecanoic, and lauric acids, which were twenty to forty times higher in LS30 than in LS25. A significant difference was also found for myristic acid, which almost matched stearic acid at 30 °C. The most abundant unsaturated FA, oleic acid, was significantly lower in LS30; also, palmitoleic acid and linolenic acid were significantly lower in LS30, with linolenic acid in LS25 more than double that in LS30.

The total unsaturated FA content was significantly higher at 25 °C than at 30 °C ($p \leq 0.05$); the total saturated FAs were higher at 30 °C than at 25 °C even if the difference was not statistically significant, and the saturated/unsaturated FA ratio was significantly

higher at 30 °C than at 25 °C (Table 3). Among the saturated FAs, long-chain FAs were similar in LS30 and LS25, while medium-chain FAs were significantly higher at 30 °C.

Table 2. SCOs' fatty acid profiles. Each fatty acid is expressed as percent of total fatty acids. LS30: fermentation carried out at 30 °C; LS25: fermentation carried out at 25 °C. Data are expressed as means of triplicate measurements. (*) indicates significant difference between treatments, (ns) indicates no significant difference following ANOVA ($p \leq 0.05$).

		Treatments		
Fatty Acid		**LS30 (%)**	**LS25 (%)**	
Caprylic acid	(C8:0)	0.4750 ± 0.15	0.0133 ± 0.02	*
Pelargonic acid	(C9:0)	0.0316 ± 0.01	0.0266 ± 0.02	ns
Capric acid	(C10:0)	1.2816 ± 0.61	0.0616 ± 0.03	*
Undecanoic acid	(C11:0)	0.0983 ± 0.03	0.0216 ± 0.01	*
Lauric acid	(C12:0)	1.7450 ± 0.99	0.0483 ± 0.04	*
Tridecanoic acid	(C13:0)	0.6250 ± 0.85	0.3183 ± 0.33	ns
Myristic acid	(C14:0)	6.7566 ± 4.01	0.9816 ± 0.35	*
Myristoleic acid	(C14:1)	1.0766 ± 0.78	0.0616 ± 0.02	ns
Pentadecanoic acid	(C15:0)	0.820 ± 0.45	0.2283 ± 0.01	ns
Palmitic acid	(C16:0)	34.025 ± 1.31	36.1483 ± 1.42	ns
Palmitoleic acid	(C16:1)	2.7316 ± 0.16	3.9366 ± 0.26	*
Heptadecanoic acid	(C17:0)	0.3333 ± 0.11	0.1416 ± 0.03	*
Heptadecenoic acid	(C17:1)	0.2783 ± 0.01	0.2933 ± 0.03	ns
Stearic acid	(C18:0)	7.7616 ± 0.89	6.480 ± 0.90	ns
Oleic acid	(C18:1)	36.566 ± 8.11	44.5033 ± 1.43	*
Linoleic acid	(C18:2)	4.4316 ± 1.11	5.5383 ± 0.20	ns
Arachidic acid	(C20:0)	0.250 ± 0.10	0.290 ± 0.06	ns
Linolenic acid	(C18:3)	0.2283 ± 0.09	0.540 ± 0.04	*
Eicosenoic acid	(C20:1)	0.1866 ± 0.07	0.0916 ± 0.02	ns
Behenic acid	(C22:0)	0.1733 ± 0.15	0.2633 ± 0.09	ns
Erucic acid	(C22:1)	0.080 ± 0.04	0	*
Lignoceric acid	(C24:0)	0.0450 ± 0.067	0.0116 ± 0.02	ns

Table 3. Total saturated and unsaturated FA composition (%) and total saturated/unsaturated FA ratio. LS30: fermentation carried out at 30 °C; LS25: fermentation carried out at 25 °C. Data are expressed as means of triplicate measurements. (*) indicates significant difference between treatments, (ns) indicates no significant difference following ANOVA ($p \leq 0.05$).

	Treatments		
Fatty Acids (%)	**LS30**	**LS25**	
Total saturated FAs	54.42 ± 8.35	45.03 ± 1.49	ns
-Medium-chain saturated FAs	11.01 ± 6.61	1.47 ± 0.61	*
-Long-chain saturated FAs	43.19 ± 2.27	43.28 ± 1.91	ns
Total unsaturated FAs	45.58 ± 8.35	54.96 ± 1.49	*
-Medium-chain unsaturated FAs	1.07 ± 0.78	0.06 ± 0.02	ns
-Long-chain unsaturated FAs	44.42 ± 9.18	54.90 ± 1.46	*
Monounsaturated FAs	40.92 ± 7.37	48.88 ± 1.57	ns
Polyunsaturated FAs	4.66 ± 1.02	6.08 ± 0.24	ns
Saturated/unsaturated FA ratio	1.25 ± 0.46	0.82 ± 0.05	*

Long-chain unsaturated FAs were significantly higher in LS25 compared to LS30; medium-chain unsaturated FAs were higher in LS30, although the difference was not statistically significant. Monounsaturated FAs were much higher than polyunsaturated FAs in both LS25 and LS30, and both mono- and polyunsaturated FAs were higher in LS25 than in LS30, although the difference was not statistically significant.

3.4. SCO Technical Properties

The biodiesel and biolubricant technical parameters estimated from the FAME profiles according to Cianchetta et al. [50] are shown in Table 4, where the limits of the U.S. biodiesel standard ASTM D6751 [51] and the E.U. standard EN 14214 [52] are also reported.

Table 4. Biodiesel technical properties calculated according to Cianchetta et al. [50]. IV = iodine value; SV = saponification value; DU = degree of unsaturation; LCSF = long-chain saturation factor; CFPP = cold filter plugging point; OS = oxidative stability; HHV = higher heating value; D = density; CN = cetane number; KV = kinematic viscosity; C18:3 = linolenic acid. ASTM D6751 and EN 14214 [51,52] are the biodiesel fuel (B 100) standards for U.S. and E.U. Peroxide values were measured. LS30: fermentation carried out at 30 °C; LS25: fermentation carried out at 25 °C. Data are expressed as means of triplicate measurements. (*) indicates significant difference between treatments, (ns) indicates no significant differences following ANOVA ($p \leq 0.05$).

	Treatments			**Standards**	
Parameters	**LS30**	**LS25**		**ASTM D6751**	**EN 14214**
IV ($gI_2/100$ g)	43.75 ± 7.8	53.22 ± 1.2	ns		<120
SV (mg KOH/g)	200 ± 4.4	197 ± 0.3	ns	370	
DU (%)	50.24 ± 9.3	61.04 ± 1.4	ns		
LCSF (%)	7.9 ± 0.1	7.56 ± 0.7	ns		
CFPP (°C)	10.46 ± 0.4	9.37 ± 2.5	ns		
OS (h)	28.81 ± 6.3	22.01 ± 0.8	ns	>3	>8
HHV (MJ/kg)	39.58 ± 0.1	39.75 ± 0	ns	44	
D (g/cm^3)	0.87 ± 0	0.87 ± 0	ns		0.86–0.9
CN	66.21 ± 0.3	65.98 ± 0.4	ns	>47	>51
KV (mm^2/s)	4.25 ± 0.1	4.45 ± 0	ns	1.9–6.0	3.5–5.0
C18:3 (%)	0.23 ± 0.1	0.54 ± 0	*		<12
Peroxide (meq O_2/kg)	0.15 ± 0.1	0	ns		

In general, the SCOs obtained at both temperatures complied with the limits for IV, SV, OS, HHV, D, KV, CN, and C18:3. A significant difference induced by the fermentation temperature was found only for the C18:3 content. Values of IV, DU, LCSF, OS, and CN were higher at 30 °C than at 25 °C. The peroxide value, indicating the degree of primary oxidation of the oil, was very low in both LS25 and LS30: 0.15 ± 0.1 meq O_2/kg at 30 °C and 0 meq O_2/kg at 25 °C.

4. Discussion

SCOs are a valuable alternative to vegetable oils to produce biodiesel and biolubricants [53,54]; however, their economic sustainability is still a limiting factor, and low-cost raw materials are necessary to boost it. Selected oleaginous yeast strains able to grow on economic substrates giving high yields are a helpful tool to reach this goal; moreover, they allow targeted products to be obtained by controlling the growth physiological conditions to direct their metabolism. Scotta is an unmarked dairy effluent suitable as a growth medium for yeasts that can use lactose as an energy and carbon source, and the high C/N ratio makes it particularly interesting for SCO production, as it is conducive to yeast lipid accumulation [23,28,55]. Nevertheless, a high C/N ratio can hinder the achievement of the high cell density that is required to reach a high lipid production rate [56].

In our assay, the pH rose during fermentation; this suggests that *L. starkeyi* DSMZ 70,295 strain can assimilate the scotta organic acids, such as lactic acid, for growth. The optimum pH for *L. starkeyi* growth is in the range between 5 and 6.5, but growth is also possible at more acidic pHs [28]. Angerbauer et al. [57] reported *L. starkeyi* growth in sewage sludge accumulating lipids in a pH range of 5.5 to 6.5; however, at pH 7.0 lipid accumulation decreased dramatically. Therefore, *L. starkeyi* can potentially grow in several wastes, such as scotta, without the need for pH control, thus avoiding the costs for pH adjustment. In our assays, the pH ranged from an initial value of 5.7 to a final

value of 6.8 in LS30 and 6.2 in LS25, so it always remained within the optimal range for growth (Figure S1). However, pH rising can hinder sugar membrane transporters and ATP expenditure in oleaginous yeasts [58]; moreover, scotta's low protein content can thwart lactose assimilation by limiting the synthesis of new membrane transporters [59]. This can explain the high residual lactose and the low biomass produced; and at the end of fermentations, indeed, cell biomass yield was lower than usually found under standard fermentations [60,61], and high residual lactose indicated the lack of its assimilation by yeast cells.

The greatest reduction in total sugars at the end of fermentation was reached in LS30, with a significant difference in comparison to LS25. The final biomass and SCO yields were higher in LS30 as well, even if the difference was not statistically significant. Yp/x and Yp/s were similar in LS25 and LS30, while Yx/s was significantly higher at 25 °C; this highlights that temperature did not significantly affect the cell lipid accumulation, but it affected sugar assimilation and cell growth. Anyway, the final SCO yield was lower than that reported for other substrates [1,19,28,44,57,60–63], but the low yields are counteracted by the lack of any additional costs for growth medium adjustments. Moreover, the worse carbon balance detected at 30 °C can be considered an additional benefit for scotta pollution load reduction, considering the high BOD and COD of this effluent [43], but mostly, the main outcome was that an SCO fatty acid profile targeted for biodiesel and biolubricants production was obtained.

The lipid profile is a key instrument to evidence the suitability of an SCO for biodiesel and biolubricant production. A high unsaturated fatty acid content affects the oxidative stability and quality of biofuels during extended storage as unsaturated molecules promote auto-oxidation; this is one main limit of vegetable oils for biodiesel production, due to their very high unsaturated fatty acid content [31]. In our assays, the peroxide values were very low, much lower than in vegetable oils [64–67], and the predicted oxidative stability values largely met the required U.S. and European standards. High-oleic oils provide oxidative stability and good cold flow properties for application as biofuel and biolubricants [68]; moreover, a very low content of linolenic acid and high content of stearic and palmitic acid are suitable for biodiesel [69]. So, the SCO composition reached in our assays can be considered highly suitable for this use. The fatty acid profile detected in both LS25 and LS30 was similar to that previously reported for *L. starkeyi* [11,28,33,44,57,62,70], with oleic acid (C18:1) and palmitic acid (C16:0) as the main fatty acids, followed by stearic (C18:0), linoleic (C18:2), and palmitoleic (C16:1) acids; linolenic acid (C18:3), whose role in biofuel quality is considered highly negative [71], was present in a very small amount and much lower than in vegetable oils [72–77]. Oils high in MUFAs (monounsaturated fatty acids) are preferred for biodiesel production because they ensure good cold flow properties, do not have a significantly negative impact on oxidative stability, and decrease NOx emissions [31]. The SCOs produced in our assays showed MUFA contents much higher than PUFAs, notably at 30 °C. Moreover, the growth temperature triggered significant further positive modification in the fatty acid profile. In LS30, significantly lower oleic and palmitoleic acid contents than in LS25 were detected. As expected, a higher temperature induced a significant increase in saturated fatty acids; however, interestingly, the increase mainly concerned shorter-chain fatty acids, such as capric, caprylic, and caproic acid, that were nearly twenty to forty times higher in LS30, and myristic acid, that almost matched stearic acid at 30 °C; total saturated medium-chain fatty acids increased almost tenfold, reaching a value of 11%. Saturated fatty acids with shorter chains can contribute significantly to maintaining a good lipid fluidity when the unsaturated fatty acid content is low [25,78,79]; so, scotta fermentation at high temperature achieved this due to a higher lipid yield, as well as having a better lipid composition, because an oil maintaining a good fluidity with a lower content of unsaturated fatty acids is also less exposed to oxidative damage. Low vulnerability to oxidative damage was confirmed by the very low peroxide values detected, lower than the vegetal or animal lipids usually used to produce biodiesel and biolubricants [80–85]. The oxidative stability inferred from the fatty acid composition [50] was very high as well.

The quality parameters of the SCOs as raw materials for biodiesel production, calculated according to Cianchetta et al. [50] and compared to the limits set by the U.S. biodiesel standard (ASTM D6751) [51] and the more stringent E.U. regulation [52] (EN 14214), confirmed the high quality of our oils for biodiesel and also for biolubricant production. The degree of unsaturation (DU) was low in both LS30 and LS25, lower than values usually reported for vegetable oils [74,76,77,86,87] and also for other SCOs [50,88,89]. This means that these oils are more stable to oxidation. The iodine value (IV), which increases with the degree of unsaturation and decreases with chain length [76], was lower than the EN 14214 limit (<120) in both LS30 and LS25, due to the low unsaturation degree and low long-chain fatty acid content; it was only slightly higher (IV 53.22 g I_2/100 g) at 25 °C, where the percentage of unsaturated fatty acids was significantly higher, compared to 30 °C (IV 43.75 g I_2/100 g), where saturated fatty acids were higher but with lower chain lengths. These characteristics are notably favorable as they are related to oils with good oxidative stability, but also good viscosity, one main factor affecting biodiesel performance in diesel engines [71,76]. The density (D), another key parameter defining fuel performance properties as it affects the mass of fuel injected into the combustion chamber, in our assays was 0.870 g cm^3, within the range between 0.860 and 0.900 g cm^3 required by EN 14214. D is correlated directly to fatty acid chain unsaturation degree and inversely to fatty acid chain length [71]. D is also related to the cetane number (CN), determined by the interval between the fuel injection and the fuel ignition, which affects the engine's cold start, stability, noise, and CO emissions: a low cetane number causes diesel knock and incomplete combustion, increasing gaseous and particulate exhaust emissions [71,90]. The CN is correlated directly to chain length and inversely to unsaturation degree [69,71]. High CN values were detected in our assays (about 66) at both temperatures, higher than most vegetable oils [76,77], slightly higher at 30 degrees due to the increased presence of saturated fatty acids. The values of linolenic acid (18:3) were much lower than the EN 14214 limits, very low in both LS30 and LS25, and lower in LS30 than in LS25. The saponification value (SV), that is inversely related to both fatty acid chain length and molecular weight, was largely below the ASTM D6751 limit of 370.

High levels of saturated fatty acids in oils have a negative impact on cold flow properties. The cold filter plugging point (CFPP), that is the temperature at which crystals in diesel fuel reach a size and quantity that may clog the fuel line and filters, is inversely related to DU. Limits are not defined by ASTM D6751 and EN 14214, as different countries may define different specifications based on national meteorological data; however, low CFPP values are mandatory in cold countries [31]. The SCOs obtained in both the LS30 and LS25 assays are not suitable for cold countries, as the CFPP values are high when compared to other vegetable or microbial oils [50,71,77,91], about 10 °C, slightly higher in LS30 due to the higher presence of saturated fatty acids. Furthermore, high levels of saturated fatty acids are related to the higher heating value (HHV), which defines the energy content released during combustion and the efficiency of a fuel; therefore, a high HHV is correlated with lower fuel consumption [90,91]. The HHV reached in both the LS30 and LS25 assays was about 39 MJ/kg, lower than the ASTM D6751 limit of 44 MJ/kg and comparable to other SCOs [50,77]. The HHV is related to viscosity; low biodiesel viscosity can cause leakage or wear of fuel injection pumps [31,71], while high viscosity can be a negative feature of biodiesel because it is not favorable to a fast atomization of the fuel spray to reduce the ignition delay period. A good biolubricant should also have the optimal viscosity to guarantee an adequate flow between metal parts [92]. The kinematic viscosity values, calculated according to Cianchetta et al. [50,93,94], were about 4.25–4.5 mm^2 s^{-1}, within the ASTM D6751 and EN 14214 standards ranges, and only slightly lower in LS30. Viscosity is correlated directly to chain length and inversely to unsaturation degree; so, the higher short- and medium-chain saturated fatty acid content was compensated by a lower unsaturated fatty acid content in the SCO produced at 30 °C, and the KV was almost equal at 25 °C and 30 °C.

Therefore, SCOs produced from fermentation of scotta by *L. starkeyi* DSMZ 70,295 can be considered suitable for biodiesel production, in particular when obtained from fermentations at 30 °C, due to their properties that ensure the optimal operating condition requirements of the compression–ignition diesel engine and an optimal oxidative stability. When compared to vegetable oils, particularly palm, coconut, and olive oil, they have similar properties for IV, CFPP, OS, HHV, CN, KV, and D, but better oxidation stability due to a lower unsaturation degree [76,91,95,96]. Good properties as biolubricants can also be inferred from their KV and D parameters. However, their cold flow properties make them unsuitable for cold climates.

5. Conclusions

Oils from microbial biomasses have several advantages over vegetable oils in the production of biodiesel and biolubricants, such as no competition for food production, no consumption of agricultural land, and no dependency on weather and seasonality. The best oil technical properties were reached in the present work by fermentation of dairy effluents inoculated with *L. starkeyi*. Moreover, as yeast lipid biosynthesis can be shifted towards a targeted fatty acid composition through the control of physiological conditions, the technical properties were further improved by control of the fermentation temperature; indeed, a significant decrease in unsaturated fatty acids, meaning a higher oxidative stability, and an increase in shorter-chain saturated fatty acids, that offset the loss of fluidity due to the lower unsaturation degree, were detected in fermentations carried out at 30 °C in comparison to 25 °C. Notably, a strong increase in myristic acid, that was seven times higher at 30 °C and almost reached the stearic acid content, and a decrease in linolenic acid, that was more than halved compared to at 30 °C, were observed. The very low peroxide values detected confirmed that these oils were strongly oxidation-resistant. So, the best oxidative stability and tribophysical properties for high-quality biolubricant and biodiesel production were reached by temperature control of the fermentation. The highest yields of biomass (9.76 g L^{-1}) and microbial oil (1.83 g L^{-1}) were also reached at 30 °C, along with a greater consumption of sugars; so, a further process benefit can come from a reduction in the pollution load of the dairy effluent used as the fermentation substrate.

The use of an effluent as the fermentation substrate without any addition or adjustment can help to reduce the process costs; however, the economic sustainability could be improved even more by increasing the fermentation yield. Further research could aim for improvement in the lactose uptake and consumption and increase the accumulation of storage lipids by *L. starkeyi* in scotta fermentations; for instance, by adjusting the substrate composition and by continuous or semi-continuous fermentation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10060278/s1, Figures S1 and S2. Figure S1. Cell growth and pH kinetics throughout the fermentations carried out at 30 °C. Data shown are means of triplicate measurements. LS30: fermentation carried out at 30 °C; CFU: colony forming units. Figure S2. Cell growth and pH kinetics throughout the fermentations carried out at 25 °C. Data shown are means of triplicate measurements. LS25: fermentation carried out at 25 °C; CFU: colony forming units.

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