Research Square

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Succinic and citric acids production from renewable biomass (waste cooking oil and crude glycerol) through oleophilic yeast strain of Yarrowia lipolytica ATCC 20177

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Abstract

Background

Organic acids like succinic and citric acids are of great interest as platform organic products that play important roles as precursors for a wide range of bio-based materials. Succinic and citric acids can be successfully produced biotechnologically from renewable resources of both hydrophilic and hydrophobic nature through efficient microbiological conversion. *Yarrowia lipolytica* represents one of the most versatile microbial factories in terms of organic acids production, as it easily develops and produced metabolites starting from glucidic-based and/or lipid-based substrates.

Results

The purpose of this work was to investigate the ability of *Y. lipolytica* to adapt to hydrophilic and hydrophobic sources and to biosynthesize important platform chemicals like succinic and citric acids. The selected strain was monitored during a batch cultivation for 192 h on 100 g/L carbon source: pure glycerol as a hydrophilic source, sunflower waste cooking oil as a hydrophobic source, and crude glycerol deriving from biodiesel production as a mixture of hydrophilic and hydrophobic sources. Cellular viability, biomass accumulation, and metabolites formation in terms of succinic acid and citric acid was monitored, and the highest results were registered for cultivations performed on waste cooking oil [10.35 ± 0.29 (log_{10}) CFU/mL, 8.15 g/L cell dry weight, 3.50 ± 0.04 g/L citric acid, and 21 ± 0.16 g/L succinic acid].

Conclusion

The results obtained in this work outline the industrial potential of the oleaginous yeast strain of *Y. lipolytica* to bioconvert lipidic residual biomass into valuable organic compounds with wide-range applicability.

Background

Succinic and citric acids represent two of the most biotechnologically produced organic acids that are largely exploited as bulk materials in different domains, starting with the bioplastics/biopolymers industries and continuing with the food, cosmetics, and pharmaceutics industries [1–4]. The global market demand for succinic acid is estimated to grow from 131.7 million USD in 2018 to 182.8 million USD by 2023 [5], while the global market for citric acid is expected to reach 3.83 billion USD by 2025 [6]. Besides their utility as platform chemicals, both organic acids are involved in the cellular metabolic processes as being intermediates of the tricarboxylic acids cycle (TCA) (also known as Krebs cycle) to provide energy [7]. So, considering these aspects, the interest in succinic and citric acid production continuously rises.

In the field of biotechnology, succinic and citric acids are mostly produced through microbial cell factories of fungal origin (e.g., *Aspergillus niger*) [8], bacterial origin (e.g., *Actinobacillus succinogenes*) [9], and yeast cells type (e.g., *Yarrowia lipolytica*) [10] by using renewable resources like those of lignocellulosic kind [11]. Biodiesel-derived glycerol is another valuable source of renewable biomass that is widely exploited as a carbon basis for the microbial production of the above-mentioned organic acids, as about 10% crude glycerol is generated as the main byproduct through the biodiesel production [12, 13]. Moreover, hydrophobic substrates like n-alka(e)nes, fatty acids, recycled oils, and greases are also an efficient source of carbon for the oleaginous microbial strains to biosynthesize succinic and citric acids [14]. Waste cooking oils for example, in the context of the last-mentioned renewable sources, are a feasible substrate for biotechnological processes that require lipidic elements like fatty acids to support the development of lipophilic microorganisms such as yeast species (e.g. *Yarrowia, Candida, Rhodosporidium, Geotrichum, Moniliella, Lipomyces, Cryptococcus*) [15–18], as over two hundred million gallons of waste oil is improperly disposed of every year [19].

Lately, multiple research studies have been conducted on *Y. lipolytica* as a robust producer of citric acid, lipases, limonene, and single-cell oils from renewable biomass derived from the agro-food sectors and biofuels industry [20–25]. This "non-conventional" strict aerobe yeast has a complex enzymatic mechanism that facilitates its great adaptability to both hydrophilic (e.g., glucose, fructose, mannose, galactose, arabinose, xylose, glycerol, etc.) and hydrophobic substrates (e.g., alka(e)nes, alkyl esters, fatty acids, triacylglycerols, etc.). As being an oleaginous microorganism, *Y. lipolytica* has the ability to catabolize lipids from the cultivation media by producing specifically targeted metabolites such as citric/isocitric acid, and at the same time, it developed the potential to biosynthesize lipidic structures such as single cell oil (SCO) from glucose or glycerol-containing substrate [26]. In addition, this strain presents good tolerance to environmental conditions in terms of salt existence, low temperatures, alkaline or acidic pH [27].

Considering all the above, and moreover the reduction of environmental pollution, for the current study, we aimed to compare and evaluate the ability of the oleaginous yeast strain (*Y. lipolytica* ATCC 20177) to bioconvert *pure glycerol* (PG) – as a hydrophilic substrate, *crude glycerol* (CG) – as a mixture of the hydrophilic-hydrophobic substrate, and *sunflower waste cooking oil* (SWCO) – as a hydrophobic substrate, into valuable bulk materials such as succinic and citric acids. SWCO played a double role in the present research work. Firstly, it was used as a lipid basis for the production of CG fraction by alkali transesterification, and secondly, SWCO has been used per se as a carbon source for the growth of *Y. lipolytica*. In addition, the content of fatty acids present in both the CG fraction and in the SWCO was established. Regarding the adaptability of *Yarrowia* cells to the hydrophilic/hydrophobic media, there were established the biomass formation, the cellular viability oscillation, the substrate consumption, and the formation of the targeted compounds (succinic and citric acids). Considering that fewer publications are available on the study of *Y. lipolytica* ATCC 20177 growth and metabolites production on different nutrient sources, in the present research paper is highlighted the *Yarrowia* cells potential to bioconvert both hydrophobic and hydrophilic carbon sources into important platform chemicals like succinic and citric acids.

Results And Discussion

SWCO and CG characterization

SWCO was collected from households and used both as lipid source for the transesterification reaction to obtain CG fraction, and also as a carbon source for the oleaginous yeast *Y. lipolytica* ATCC 20177 during the fermentation processes. Before being used for CG production or batch fermentation, SWCO was separated from the coarse parts by cloth filtration. The transesterification process consisted of mixing the SWCO with the methoxide solution (MeOH + NaOH) and letting it rest for about 48 h, from where the majority of formed methyl esters of the fatty acids were separated on the superior part of the funnel, forming the biodiesel fraction. The unreacted fatty acids, unreacted methanol, glycerol, water, catalyst, and other unreacted molecules present in SWCO have accumulated in the CG fraction, which was separated at the inferior part of the funnel [28–30]. Before and after the transesterification, the content of fatty acids in SWCO and CG fraction was established by GC-MS and is presented in Table 1. The pH of the resulted CG was around 9. As it can be observed, SWCO showed increased values for palmitic acid, oleic acid, and linoleic acid, for which were recorded about 9%, 41%, and 75%, respectively. After the alkali transesterification of SWCO, the content of mentioned fatty acids in CG decreased to 0.67%, 24%, and 45.23%, respectively, aspect generated by the fatty acids methyl esters formation (e.g., methyl palmitate, methyl oleate, or methyl linoleate) in the upper biodiesel fraction [31, 32]. The percentages of the major fatty acids identified in SWCO were slighty higher compared to other studies where palmitic acid recorded 8.90%, oleic acid was 30.71%, and linoleic acid was 54.35% [33]. Similar results were obtained for oleic and linoleic acids found in CG compared with the scientific literature (23.2% and 46.8%), while lower values were recorded in the case of palmitic acid (10%) [34].

Table 1
The FFA profile in SWCO and CG obtained by the alkali transesterification of SWCO, the glycerol content and the pH of CG fraction.

Fatty Acids	**SWCO (%, ±)**	**CG (%, ±)**
(16:0) Palmitic acid	9.22 ± 0.02	0.67 ± 0.01
(18:0) Stearic acid	2.55 ± 0.00	0.63 ± 0.00
18:1n-9 Oleic acid	41.05 ± 2.71	23.81 ± 0.51
18:1n-7 Vaccenic acid	0.7 ± 0.00	0.16 ± 0.00
18:2n-6 Linoleic acid	74.75 ± 2.22	45.23 ± 1.75
18:3n-3 α-Linolenic acid	0.03 ± 0.00	-
(20:0) Arachidic acid	0.01 ± 0.00	-
(22:0) Behenic acid	-	0.01 ± 0.00
TOTAL Fatty Acids		
SFA	11.78 ± 0.02	1.31 ± 0.01
MUFA	41.75 ± 2.71	23.97 ± 0.51
PUFA	74.78 ± 2.22	45.23 ± 1.75
n-3 PUFA	0.03 ± 0.00	-
n-6 PUFA	74.75 ± 2.22	45.23 ± 1.75
pH	-	9.03 ± 0.02
Glycerol content (%)	-	30 ± 1.05

The type of greases and waste vegetable oils resulting from the food processing industry collected for biodiesel manufacturing plays an important role in the conversion yield through transesterification. The presence of different impurities such as minerals, organic matter, or water decreases the overall quality and productivity of the biodiesel fraction [28, 29]. In this regard, the conversion yield of lipids into fatty acids methyl esters can be established with precise analytical methods, like FT-IR.

FT-IR is a frequently used instrument for identifying molecular bonds among different functional groups within a specific matrix. Also, in this experimental work was established the FT-IR fingerprint for SWCO, unpurified CG, and PG as three different carbon sources used for the growth of *Yarrowia* cells. From Fig. 1, the spectra for the analyzed samples are clearly different, as they overlap only at some specific absorbance units. As a result, bending (scissoring and rocking) vibrations specific to the C-H group were observed at 721 cm^{-1}, 1375 cm^{-1}, and 1462 cm^{-1} for SWCO; at 719 cm^{-1}, 1361 cm^{-1}, and 1460 cm^{-1}

for CG, while for PG the bands at the similar absorbances are hardly identifiable. In both SWCO and CG samples, multiple high-pitched peaks indicating C-O streching are observed between 1030–1500 cm^{-1} (specifically 1031/1037 cm^{-1}, 1118/1111 cm^{-1}, 1161/1170 cm^{-1}, 1462/1460 cm^{-1} for SWCO/CG), while for PG a pronounced peak associated with C-O vibrations is noticed at 1029 cm^{-1} [35, 36]. The large versatility of the peaks identified at 700–1700 cm^{-1} in CG especially, is due to the transesterification that releases the fatty acids from the structure of the triglycerides. This fact can be correlated with the presence of the sharp peak at 1560 cm^{-1} in CG (otherwise missing from both SWCO and PG), which is further linked with the vibrations of COO^- groups particular to the catalyst involved in the transesterification reaction [30]. Moreover, in the case of SWCO and CG, it was noticed a strong absorption band of the carbonyl group (C = O) between 1735–1750 cm^{-1} (specifically at 1743 cm^{-1} for SWCO and 1741 cm^{-1} for CG), a band that misses for PG, and which is associated with the presence of fatty acids [35]. According to Matwijczuk and co-workers (2018), the vibrations close to 2850 cm^{-1}, 2870 cm^{-1}, and 2925 cm^{-1} are characteristic to the hydrocarbon chain bands, precisely to methyl ($-CH_3$), methylene ($-CH_2-$), and C-H groups [35]. So, in our case, SWCO illustrated sharp peaks at 2852–2922 cm^{-1}, CG showed high peaks at 2852–2924 cm^{-1}, while PG presented moderate absorbance bands at 2879–2933 cm^{-1}. The small peaks observed at 3005 cm^{-1} and at 3008 cm^{-1} in SWCO and in CG (and absent in PG) point to the presence of = C-H vibrations specific to the olefins [37]. The functional hydroxyl groups (-OH) that indicate the presence of polyalcohols (glycerol in this case) are confirmed by the large peak identified at 3358 cm^{-1} in CG and 3264 cm^{-1} in PG, and which are entirely missing from the SWCO spectra.

Y. lipolytica's ability to biosynthesize citric and succinic acids from different carbon sources

Y. lipolytica is considered one of the most efficient oleaginous platform microorganisms that can produce both fatty acids-derived biochemicals and bio-combustibles [38]. Most of the organic acids produced by oleaginous strains like *Y. lipolytica* are generated as additional metabolites and excreted outside the cell during the *de novo* and *ex novo* fatty acids metabolic production (Fig. 2) [16].

By *de novo* pathway, the yeast cells use glycolytic reactions to consume hydrophilic substances like glucose, acetate, or glycerol to biosynthesize different lipid structures (ex. C_{14}, C_{18}), which accumulate inside the cells. When glycerol is used a sole nutrient, which is partly the case of the present study, the yeast cell converts it to pyruvic acid through a series of biochemical reactions (where glycerol-3-phosphate, and dihydroxiacetonepyruvate phosphate intermediate these reactions) that takes place inside the cytosol. Further, the obtained pyruvate is moved to mythocondria where is bioconverted into acetyl-coA, one the most important substrates that triggers the formation of organic acids via Krebs cycle (TCA cycle) [18, 39, 40]. On the other hand, by *ex novo* pathway, *Yarrowia* cells use hydrophobic sources

(e.g., alka(e)nes, fatty acids, fatty acids esters, triacylglycerol, etc.) for specific oxidation processes from where dicarboxylic acids can occur (e.g., succinic acid), or for accumulation within the cell body [18].

Y. lipolytica accumulates lipids inside the body cell for biomass formation and to generate novel fatty acids. As more the lipids or the hydrophobic sources are assimilated by the yeast body cells on the *ex novo* pathway, the higher the amount of dicarboxylic acids (e.g., succinic acid) are excreted in the culture medium, as could be observed from results illustrated in Fig. 3 and Supplementary materials S1. In the current work, the highest values in terms of yeast's cellular viability [10.35 ± 0.29 (log_{10}) CFU/mL], biomass formation (8.15 ± 0.23 g/L), and metabolites generation (citric acid: 3.50 ± 0.04 g/L, succinic acid: 20.99 ± 0.16 g/L) after 192 h of batch fermentation were achieved when hydrophobic nutrients like SWCO were used as feeding source (Fig. 3C) compared with glycerol (PG, CG) as the main nutrient substrate (Fig. 3A, Fig. 3B). Apparently, the *Y. lipolytica* ATCC 20177 yeast cells adapted their metabolism much easier to the hydrophobic sources than to hydrophilic ones, as resulted from their viability that increased up to 85% when cultivated on SWCO, compared with 42% when cultivated on CG, and with 11% when cultured on PG. Their viability in PG-containing medium maintained constant values during the process, while in CG-containing medium (when hydrophobic structures are available) the viability and biomass slightly increased along the cultivation process. As CG consists of a mixture of hydrophilic and hydrophobic carbon sources, both *de novo* and *ex novo* metabolic pathways are stimulated, so in this case, glycerol is used by the cell to produce energy inside the mitochondria that generates further the citrate and succinate extracellular excretion, while the fatty acids accumulate inside the cell triggering the biomass formation and the intracellular lipids [2, 41, 42].

The results reported in the present work considering the organic acids production are close to those reported by Terboven and colleagues (2021). They obtained a maximum concentration of 23 g/L succinic acid by fermenting lactose concentrate from cheese production with dedicated bacterial strains like *Actinobacillus succinogenes* 130Z (DSM 22257) and *Basfia succiniciproducens* DSM 22022 [38]. Patel and Matsakas (2019) investigated the metabolic potential of hydrophobic sources like rapeseed waste cooking oil by an oleaginous yeast strain named *Cryptococcus curvatus* DSM-101032 into a batch cultivation, in parallel with trials containing glucose as the hydrophilic source. At the same time, the authors used ultrasonicated and unsonicated oil for the cultivation media, and evaluated the biomass evoluation and metabolites formation (e.g. extracellular lipids). The main results of that study pointed out the *ex novo* assimilation potential of the hydrophobic sources by the yeasts cells recording the highest extracellular lipid content of 70.13 ± 1.65% and 13.06 ± 0.92 g/L of cell dry weight, when grown on 20 g/L ultrasonicated waste cooking oil. In parallel, through the *de novo* pathway, significantly inferior values were recorded for both the extracellular lipids (52.66 ± 0.93%) and for the cell dry weight (9.93 ± 0.84 g/L) when 40 g/L of glucose were used [16]. Moreover, in a research study performed by Gao and colaborators (2016) on a mutant strain of *Y. lipolytica* PGC01003 was achieved 43 g/L succinic acid from 200 g/L crude glycerol (which equivalents 129.4 g/L pure glycerol) during batch cultivation at pH 6, and up to 160 g/L succinic acid during a fed-batch fermentation on CG [27].

The outcomes of this work highlight the industrial potential of *Y. lipolytica* yeast strain ATCC 20177 as this can metabolize hydrophobic sources like residual cooking oil and CG deriving from the biofuels industry transforming them into valuable platform chemicals. Furthermore, *Y. lipolytica* owns adaptation mechanisms to glycerol-containing medium and fatty acids being stimulated *de novo* and *ex novo* pathways, which results in organic acids extracellular excretion and elevated biomass formation.

Conclusions

Renewable biomass like crude glycerol and waste cooking oil was used as a carbon basis for the biosynthesis of platform organic acids like succinic and citric acids, through oleophilic yeast strain, namely *Yarrowia lipolytica* ATCC 20177. Pure glycerol was used as a sole hydrophilic source for stimulating *de novo* metabolic pathway; the waste cooking oil represented the hydrophobic source for stimulating *ex novo* metabolic pathways in the yeast cells; crude glycerol consisted of a mixture of both hydrophilic and hydrophobic carbon sources. The used yeast strain gave increased results when cultivated on waste oil compared with glycerol (pure or crude), fact that supports the idea of an efficient microbial convertor for waste biomass of lipidic nature into important bulk chemicals such as organic acids (succinic and citric acids).

Materials And Methods

Regeants used for the experimental work, were analytical grade purchased from Sigma-Aldrich (Sigma-Aldrich Trading Co., Shanghai, China). The renewable nutrient substrate was SWCO obtained from households (Cluj-Napoca, Romania). SWCO was used by itself as a carbon source for microbial growth, and it was also used as a lipidic substrate for transesterification reaction to obtain CG fraction.

CG obtaining process (transesterification)

CG fraction was obtained from SWCO in our laboratory using alkali transesterification, as follows: 1000 mL of SWCO were mixed with 240 mL of anhydrous methanol 99.8% and 7 g of NaOH (reagent grade, ≥ 98%, anhydrous pellets), for 24–48 h at room temperature. Two independent phases were obtained: 1030 mL of upper phase - fatty acids methyl esters (biodiesel fraction), and 100 mL of lower phase - aqueous phase (CG fraction) [30, 43, 44]. The content of glycerol, pH, and fatty acids profile were analyzed further for the CG phase. Before adding to the cultivation media, the pH of CG was adjusted to 6 with 2M HCl, the methanol content was removed by evaporation at 70°C for about 40 min (Heidolph Laborata 4000 rotary evaporator, Heidolph Instruments, Schwabach, Germany), and it was sterilized separately at 120°C for 20 min.

Microorganism and culture media:

Lyophilized *Y. lipolytica* ATCC 20177 purchased from American Type Culture Collection (Manassas, Virginia, USA) was used for the experimental trials. The cells were maintained on yeast malt extract agar

plates (yeast extract 3 g/L, malt extract 3 g/L, dextrose 10 g/L, peptone 5 g/L, agar 20 g/L) at 4°C, and re-activated periodically at every 2–3 months.

Culture media components and cultivation conditions were similar to those mentioned by Anastassiadis and Rehm [45]. The inoculums representing 10% of the culture were prepared by transferring 10^7 CFU/mL (corresponding to 20 mL cellular suspension) into 500 mL shake flask with buffers containing 200 mL of fresh culture media with the following components: 6 g/L NH_4Cl, 1.05 g/L KH_2PO_4, 1.48 g/L $MgSO_4$ x $7H_2O$, 0.33 g/L $MnSO_4$ x $4H_2O$, 0.14 g/L $FeSO_4$ x $7H_2O$, 4 mg/L $CuSO_4$ x $5H_2O$, 0.08 g/L $ZnSO_4$ x $7H_2O$, 8 mg/L $CoSO_4$ x $7H_2O$, 0.08 g/L H_3BO_3, 0.2 g/L $CaCl_2$, 0.2 g/L NaCl, 0.2 mg/L KI, 0.4 mg/L Na_2MoO_4 x $2H_2O$, 0.5 g/L citric acid, 4 mg/L Thiamine-HCl, 0.5 mg/L Biotin, 1.25 mg/L Pyridoxine-HCl, 1.25 mg/L Ca-D-Pantothenate, 1 mg/L Nicotinic acid. The carbon sources used for the inoculums were 50 g/L of PG, CG, and SWCO. The shake flasks were incubated for 2 days at 30°C, pH 5, and 200 rpm (Innova 44 Incubator Shaker, New Brunswick, Eppendorf, Hamburg, Germany).

For the batch fermentations at the bioreactor level (2 L working volume) was used cultivation media consisting of the following components: 6 g/L NH_4Cl, 100 g/L carbon source (PG, CG, SWCO), 1.05 g/L KH_2PO_4, 1.48 g/L $MgSO_4$ x $7H_2O$, 0.33 g/L $MnSO_4$ x $4H_2O$, 0.14 g/L $FeSO_4$ x $7H_2O$, 4 mg/L $CuSO_4$ x $5H_2O$, 0.08 g/L $ZnSO_4$ x $7H_2O$, 8 mg/L $CoSO_4$ x $7H_2O$, 0.08 g/L H_3BO_3, 0.2 g/L $CaCl_2$, 0.2 g/L NaCl, 0.2 mg/L KI, 0.4 mg/L Na_2MoO_4 x $2H_2O$, 2.5 g/L citric acid, 4 mg/L Thiamine-HCl, 0.5 mg/L Biotin, 1.25 mg/L Pyridoxine-HCl, 1.25 mg/L Ca-D-Pantothenate, 1 mg/L Nicotinic acid. Sterile silicon oil was periodically added as antifoaming agent. Vitamins (thiamine-HCl, biotin, pyridoxine-HCl, Ca-D-Pantothenate, and nicotinic acid) and the ammonium source (NH_4Cl), for both inoculums and batch fermentation media, were added separately after the medium autoclaving, by filtration through 0.20 µm sterile filters (Macherey-Nagel, Düren, Germany).

Bioreactor batch cultivation:

All experiments were conducted in a 5 L Eppendorf bioreactor (model: BioFlo® 320, one unit, Eppendorf, Hamburg, Germany) filled with a 2 L work volume of medium culture connected and equipped with pH and temperature sensors. Temperature, pH, and rotation were maintained at 30°C, 5, and 200 rpm, and the pH was adjusted automatically by adding 40% NaOH. The inoculums were added in sterile conditions. The fermentation process ran for 192 h in aerobic conditions, maintained by continuous addition of filtered air (0.20 µm filters - Macherey-Nagel) into the fermentation broth through a peristaltic pump (Watson Marlow 520 S, Cornwall, England) settled at 10 rpm and 200 mL/min. Periodically, samples were collected to perform specific tests.

Analytical assays

Fatty acids profile from SWCO and CG

The fatty acids concentration from SWCO and CG was established by Gas Chromatography - Mass Spectrometry (GC-MS). The profile of the fatty acids from the total lipids was determined through transesterification by using 1% H_2SO_4 in methanol [30, 46]. The methylated fatty acids content was determined with a gas chromatograph coupled to a mass spectrometer, model PerkinElmer Clarus 600 T GC-MS (PerkinElmer, Inc., Shelton, CT, USA). A volume of 0.5 µL sample was injected into a 60 m × 0.25 mm i.d., 0.25 µm film thickness SUPELCOWAX 10 capillary column (Supelco Inc., Darmstadt, Germany). The procedure was conducted under the following conditions: injector temperature 210°C; as carrier gas was used helium at a flow rate of 0.8 mL/min; the split ratio of 1:24; oven temperature 140°C (hold 2 min) to 220°C at 7°C/min (hold 23 min); electron impact ionization voltage was 70 eV; trap current 100 µA; ion source temperature 150°C; mass range 22–395 m/z (0.14 scans/s with an intermediate time of 0.02 s between the scans). The fatty acids content was identified by comparing their retention times with known standards (37 components FAME Mix, Supelco no. 47885-U, Darmstadt, Germany) and the resulting mass spectra to those in the database (NIST MS Search 2.0). The amount of each fatty acid was expressed as a percentage of total fatty acid content.

FT-IR fingerprint

Fourier Transform Infrared Spectrophotometry (FT-IR) was used to observe the fingerprint for the molecular vibrations of PG, CG, and SWCO. In this regard, an FT-IR spectrophotometer (model: Shimadzu IR Prestige – 21) was equipped with an attenuated total reflectance module, and as background, petroleum ether was used. The spectra were recorded on a wavelength range of 600–4000 cm^{-1} at a resolution of 4 cm^{-1}, and 64 scans for a spectrum [29].

Biomass, cellular viability, viability staining:

Biomass formation and cellular viability were determined as in our previous work [30]. Biomass as the quantity of Cell Dry Weight (CDW) was established by filtering 10 mL of the fermentation broth through 0.20 µm filters, washing them twice with double distilled water, and drying them at 104°C for 8 h until a constant mass was observed.

The cellular viability was determined by diluting 1 mL of fermentation sample to 9 mL of a sterile saline solution of 0.8% NaCl. A volume of 100 µL from different dilutions was passed on Petri dishes with yeast malt extract agar and incubated at 30°C for about 2 days. The plate counting method [47] established the viability of *Y. lipolytica* cells (log_{10} CFU/mL). The cellular viability was also observed under the microscope light as methylene-blue colored cells, while colorless cells were considered unviable [48].

Succinic and citric acids production, and substrate consumption (PG, CG, SWCO)

The succinic and citric acids content and the consumption of glycerol from the cultivation media were identified through HPLC [49]. The HPLC unit was equipped with a quaternary pump, solvents degasser, and manual injector coupled with a refractive index detector (RID) (Agilent 1200, Santa Clara, CA, USA). The compounds separation was performed using a Polaris Hi-Plex H column, 300 × 7.7 mm (Agilent

Technologies, CA, USA). As mobile phase was used 5 mM H_2SO_4 with a 0.6 mL/min flow rate. The column temperature was 80°C, and the RID temperature was maintained at 35°C. The elution of the compounds runs for 20 min. The data acquisition and the interpretation of the results were performed using OpenLab CDS ChemStation Edition software (Agilent Technologies, CA, USA).

The consumption of fatty acids from the cultivation media consisting of SWCO was monitored by GC-MS, through the method described previously [30, 46].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

Laura Mitrea: Conceptualization, Formal analysis, Investigation, Writing - original draft. **Lavinia-Florina Călinoiu**: Writing - original draft, Investigation. **Bernadette-Emőke Teleky**: Formal analysis, Investigation. **Katalin Szabo**: Conceptualization, Formal analysis. **Adrian-Gheorghe Martău**: Investigation; Methodology. **Bianca-Eugenia Ştefănescu**: Validation, Writing - review & editing. **Francisc-Vasile Dulf**: Formal analysis,

Investigation, Resources. **Dan-Cristian Vodnar**: Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

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Figures

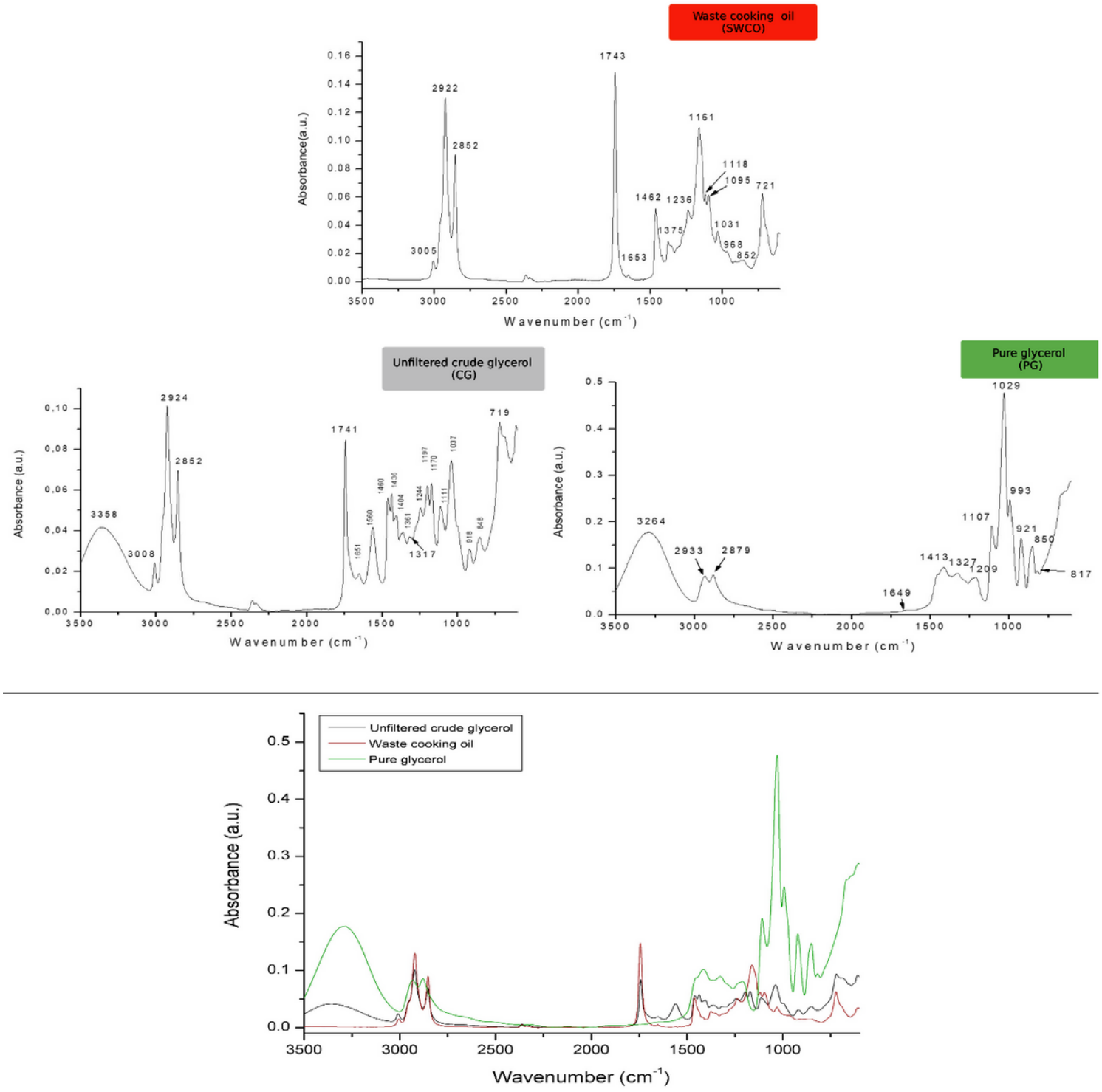

Figure 1

The FT-IR finger print for sunflower waste cooking oil (SWCO), crude glycerol (CG), and pure glycerol (PG).

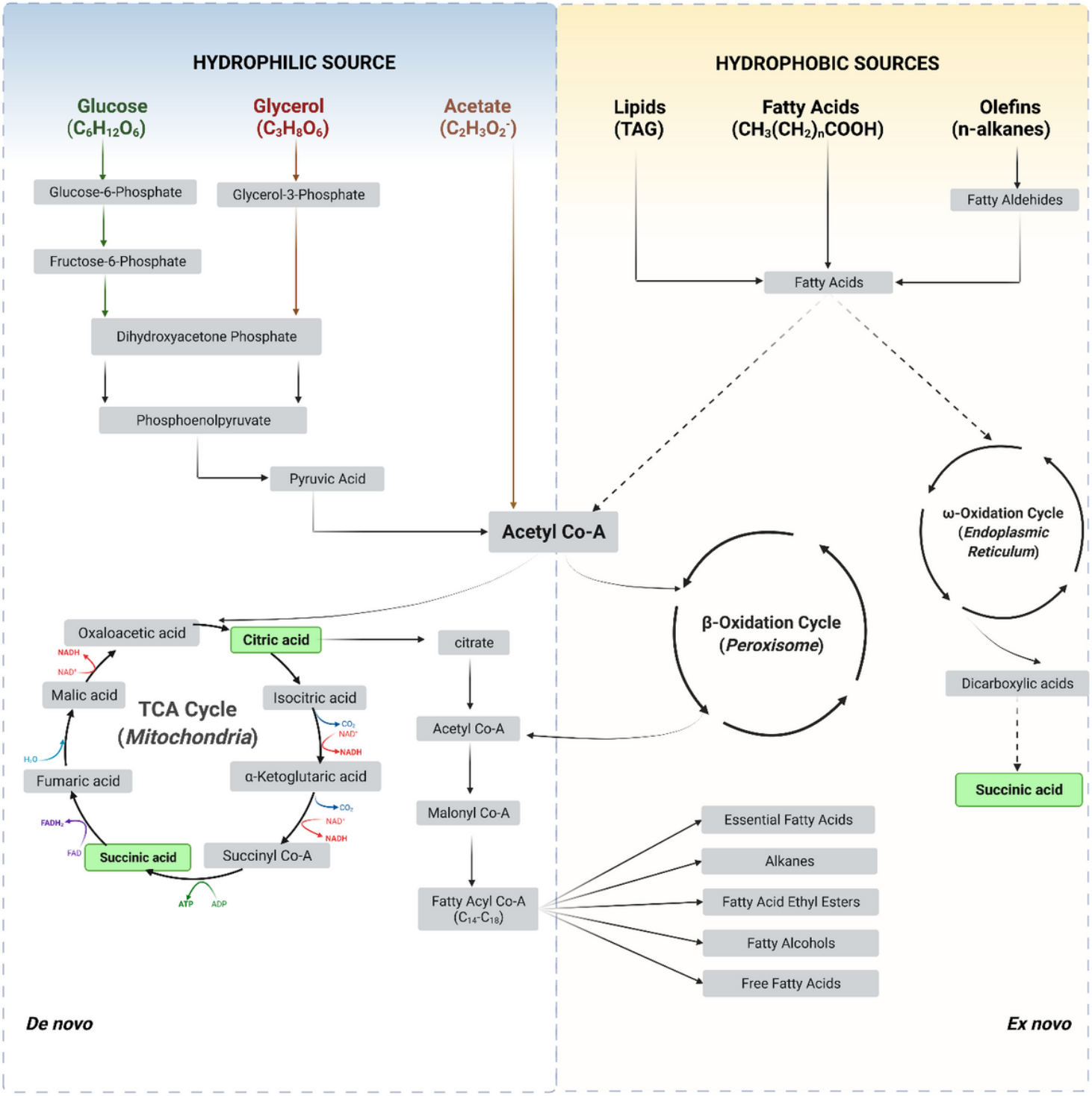

Figure 2

De novo and *ex novo* pathways used by used by *Yarrowia lipolytica* to metabolize hydrophilic and hydrophobic carbon sources. Adapted after (Chattopadhyay et al., 2021; Liu et al., 2021; Patel et al., 2020; Patel & Matsakas, 2019).

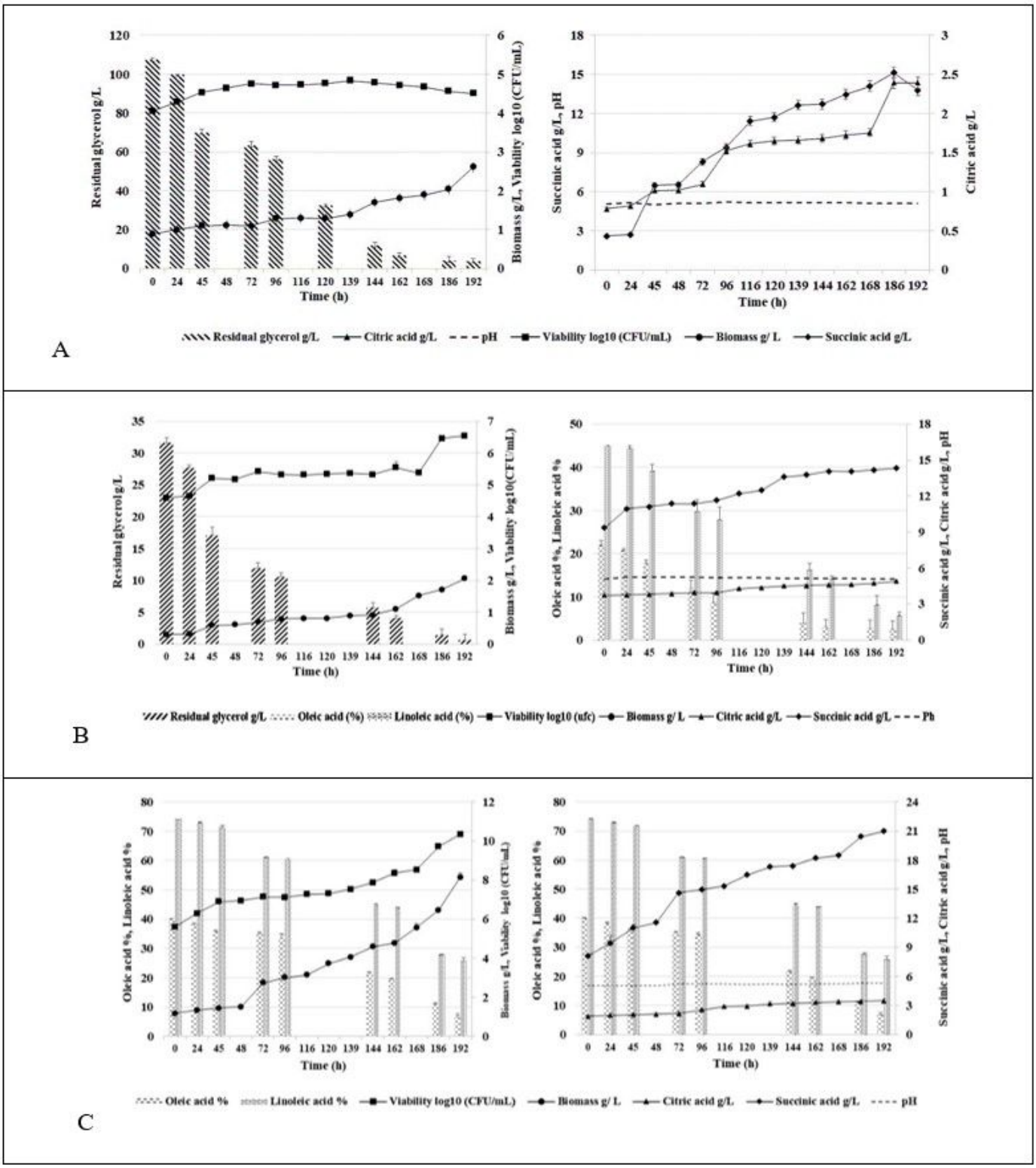

Figure 3

The results obtained with *Y. lipolytica* cultivated for 192 h on (A) - Pure Glycerol (PG), (B) - Crude Glycerol (CG), and (C) - Sunflower Waste Cooking Oil (SWCO). The results are given as mean values of two replicates (standard deviations SD under 5%).

Supplementary Files

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