Yarrowia lipolytica as a biorefinery platform based on low-cost feedstocks

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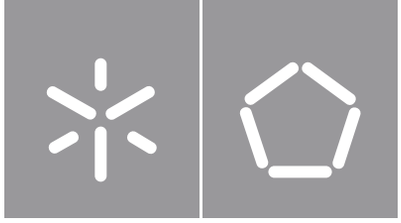

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Work developed under the supervision of:
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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism, any form of undue use of information, or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Yarrowia lipolytica como plataforma de biorrefinaria a partir de matérias-primas de baixo custo

Atualmente, o biodiesel com origem nos lípidos microbianos tem atraído muita atenção como alternativa aos combustíveis fósseis. No entanto, os preços elevados dos substratos puros usados na produção de lípidos microbianos limitam a sua aplicação. A levedura oleaginosa *Yarrowia lipolytica* é considerada interessante para a produção sustentável de lípidos a partir de matérias-primas de baixo custo, uma vez que é capaz de utilizar diversos substratos, como por exemplo os ácidos gordos voláteis (AGVs) derivados de resíduos orgânicos.

Assim, inicialmente, foi avaliada a capacidade de *Y. lipolytica* W29 e *Y. lipolytica* NCYC 2904 de crescerem e acumularem lípidos a partir de um meio sintético contendo AGVs. *Yarrowia lipolytica* cresceu neste meio, contudo a produção de lípidos aumentou com adição de co-substratos (glucose ou glicerol) em culturas descontínuas. Foi demonstrado que a transferência de massa de oxigénio é um fator importante para a produção de lípidos por *Y. lipolytica* a partir de AGVs. Além disso, a cultura em duas fases descontínuas, *two-stage batch* (TSC) - fase de crescimento em glucose, seguida por adição de 18 $g \cdot L^{-1}$ de AGVs - foi a melhor estratégia para obter o maior conteúdo em lípidos (25,4 % p/p para *Y. lipolytica* W29 e 26,7% p/p para *Y. lipolytica* NCYC 2904). A cultura TSC em biorreator, a operar com 40 % a 50 % da saturação de oxigénio dissolvido durante a fase produção de lípidos, resultou num aumento de 1,5 vezes no conteúdo de lípidos de *Y. lipolytica* NCYC 2904. Usando meio fermentado contendo AGVs produzidos durante a fermentação acidogénica de resíduos alimentares foram obtidos 11 $g \cdot L^{-1}$ de lípidos em cultura TSC com fase de crescimento no meio fermentado com AGVs, seguida pela produção de lípidos em glicerol bruto (subproduto da indústria de biodiesel). Este processo biotecnológico permitiu a integração de um processo anaeróbio e aeróbio criando uma cadeia de valor desde resíduos e subprodutos até a produção de lípidos microbianos. Estes são compostos pelos ácidos palmítico, palmitoleico, esteárico, oleico e linoleico, semelhantes aos óleos vegetais, tornando-os matérias-primas adequadas para a produção de biodiesel. Estes lípidos microbianos foram usados com sucesso na produção de biodiesel, cujas propriedades atendem aos critérios dos padrões internacionais.

Palavras-chave: Biodiesel, glicerol bruto, lípidos microbianos, *Yarrowia lipolytica*, ácidos gordos voláteis

ABSTRACT

Yarrowia lipolytica as a biorefinery platform based on low-cost feedstocks

Nowadays, microbial lipids-derived biodiesel is garnering much attention owing to its potential to substitute diesel fuel. However, the high costs of pure substrates used in microbial lipids production limit their application. The oleaginous yeast *Yarrowia lipolytica* is considered interesting for the sustainable production of microbial lipids from low-cost feedstocks since it can use a wide range of substrates, such as volatile fatty acids (VFAs) derived from organic wastes

Thus, firstly, the ability of *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 to grow and accumulate lipids intracellularly using a synthetic medium containing VFAs was evaluated. *Yarrowia lipolytica* grew efficiently in VFAs-based media, but lipids production was enhanced by the addition of co-substrates (glucose or glycerol) in batch cultures. It was demonstrated that oxygen mass transfer is an important factor for lipids production by *Y. lipolytica* from VFAs. Moreover, a two-stage batch culture (TSC) – growth phase in glucose, followed by the addition of 18 $g \cdot L^{-1}$ VFAs - was the best strategy to obtain the highest lipids content (25.4 % w/w in *Y. lipolytica* W29 and 26.7 % w/w in *Y. lipolytica* NCYC 2904). The TSC in a lab-scale bioreactor with 40 % to 50 % of saturation of dissolved oxygen during the lipogenic phase resulted in a 1.5-fold improvement in lipids content by *Y. lipolytica* NCYC 2904. Using VFAs-rich fermented medium generated during the acidogenic fermentation of food waste (FW), 11 $g \cdot L^{-1}$ lipids were obtained in a TSC strategy where the growth phase in FW-derived VFAs was followed by the lipogenic phase in crude glycerol (by-product of the biodiesel industry). This biotechnological process allowed the integration of an anaerobic and aerobic process to generate a value chain from wastes and by-products to microbial lipids production. Microbial lipids produced by *Y. lipolytica* are composed of palmitic, palmitoleic, stearic, oleic, and linoleic acids, a similar composition to vegetable oils, making them suitable feedstocks for biodiesel production. These microbial lipids were used to successfully produce biodiesel, whose properties meet the criteria of the international biodiesel standards.

Keywords: Biodiesel, crude glycerol, microbial lipids, *Yarrowia lipolytica*, volatile fatty acids

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LIST OF ABBREVIATIONS

ACS	Acetyl-CoA synthase
AD	Anaerobic digestion
ANOVA	One-way analysis of variance
ASTM	American Society for Testing and Materials
ATP	Adenosine triphosphate
CFPP	Cold filter plugging point
C/N	Carbon/nitrogen ratio
CN	Cetane number
COD	Chemical oxygen demand
DNS	3,5-dinitrosalicylic acid
EN	European Standard
EU	European Union
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FID	Flame ionized detector
FW	Food waste
GC	Gas chromatography
HHV	High heating value
HPLC	High-performance liquid chromatography
HRT	Hydraulic retention time
k_La	Oxygen volumetric mass transfer coefficient
LCFA	Long-chain fatty acids
MUFA	Monounsaturated fatty acids
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate buffered saline
PPP	Pentose phosphate pathway
PUFA	Polyunsaturated fatty acids
$R_{glucose}$	Glucose uptake rate
RI	Refractive index detector
rpm	Rotations per minute
R_{VFA}	Volatile fatty acids uptake rate

SCO	Single-cell oil
SDGs	Sustainable Development Goals
SFA	Saturated fatty acids
STR	Stirred tank reactor
TAG	Triacylglycerols
TCA	Tricarboxylic acid cycle
TN	Total nitrogen
TOC	Total organic carbon
UV	Ultra-violet detector
VFAs	Volatile fatty acids
VS	Volatile solids
vvm	Volume of air per volume of medium per minute
$Y_{L/S}$	Lipids yield
YNB	Yeast Nitrogen Base medium
YPD	Yeast extract, peptone, dextrose medium
$Y_{X/S}$	Biomass yield

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CHAPTER 1

Motivation and outline

1.1. Contextualization and motivation

Global energy crisis and environmental concerns are currently among the main challenges to address in order to meet the Sustainable Development Goals (SDGs) of the United Nations. About 70 % of energy production in the European Union still comes from fossil fuels, which contributes to climate change and global warming (Holechek et al. 2022; Eurostat 2023a) The imminent exhaustion of fossil energy resources and the problems posed to the environment led to the need of a global energy transition from fossil fuel to renewable and environmentally friendly energy. European Union (EU), as part of the European Green Deal and REPowerEu plan, sets a renewable energy target of at least 42.5 % of total energy consumption by 2030 (European Commission 2023a).

In a similar trend, the EU has been implementing waste management policies to reduce the environmental impacts of waste and improve resource efficiency. The long-term aim of these policies is to improve waste management, stimulate recycling and limit the use of landfilling (European Commission 2023b). In 2020, 2.15 billion tonnes of waste were generated in the EU, and about 39.2 % of waste was recycled and 32.2 % went to landfill (Eurostat 2023b). Landfilling is the least preferable option of waste management due to being considered harmful to the environment through the greenhouse gases emission from its decomposition and water and soil pollution (Tenodi et al. 2020; Usmani et al. 2021). Thus, proper waste management is vital to mitigate further environmental impact and promote the world's transition to energy sustainability. Developing systems for the sustainable use of waste through the biorefinery concept is a promising approach to consider waste-as-a-value, contributing to proper and green waste management.

Biorefinery is defined as "the sustainable processing of biomass into a spectrum of markable products and energy" (IEA Bioenergy 2023). The microbial bioconversion process is the most flexible biorefinery technology for converting waste biomasses into a wide variety of bioproducts that can be used as building blocks for the production of biofuels, chemicals or other products of industrial interest. Oleaginous yeasts can be a suitable option to valorise waste as feedstock for valuable bioproducts (Gottardi et al. 2021; Caporusso et al. 2021). Microbial lipids (also called single-cell oils or bio-oil), produced by oleaginous yeast, have attracted a great deal of attention over the past years as a potential feedstock to replace vegetable oils in biodiesel manufacturing since its composition is similar to common vegetable oils. Microbial lipids have advantages over vegetable oils in terms of productivity, site and labour requirements, feasibility of scale-up and life cycle (Szczepańska et al. 2021). The use of low-cost substrates, such as waste or renewable feedstocks, is of utmost importance for the economic and competitive production of microbial lipids.

Yarrowia lipolytica, a model oleaginous yeast, can be used as a biorefinery platform due to its outstanding ability to use a variety of substrates. Moreover, *Y. lipolytica* has been considered an industrial workhorse due to its ability to produce important metabolites, an intense secretory activity and efficient system for genetic engineering transformation (Coelho et al. 2010; Liu et al. 2015). Under specific cultures conditions, *Y. lipolytica* can accumulate lipids at more than 20 % of their dry weight, which are a promising feedstock for biodiesel production.

The main goal of this thesis is the development of bioprocesses based on food waste for the production of microbial lipids, exploring the abilities of *Y. lipolytica* to grow in a wide range of substrates and to produce a wide range of products of industrial interest. Given the complex matrix of food waste, anaerobic digestion (AD) has been applied as a pretreatment of food waste, converting them to volatile fatty acids (VFAs), which can be further used as carbon source by *Y. lipolytica*. The possibility of VFAs bioconversion generated from organic waste to microbial lipids by *Y. lipolytica* was accessed since it seems a feasible strategy for cost-effective microbial lipids production and could also contribute to develop a more attractive alternative to the valorization of the organic waste than the traditional waste management. Thus, an eco-friendly and sustainable process was developed to obtain raw-material for biodiesel production, favoring the Circular Economy and contributing for the advancement of the SDGs, in particular, SDG 7 (affordable and clean energy), SDG 12 (responsible consumption and production) and SDG 13 (climate action).

For this purpose, this thesis comprises several specific goals:

- Evaluation of the ability of two *Y. lipolytica* strains to grow and accumulate lipids intracellularly using VFAs as carbon source.
- Optimization of cellular growth and lipids accumulation from VFAs selecting key operating factors (co-substrates, operation mode, and oxygen).
- Validation of *Y. lipolytica* growth and intracellular lipids accumulation in a medium containing VFAs produced during the acidogenic fermentation of organic waste samples.
- Scale-up from flasks to bioreactors of the bioprocess of *Y. lipolytica* lipids production. Comparison of batch and two-stage batch modes of operation.
- Characterization of microbial lipids produced and estimation of its potential application on biodiesel production.

1.2. Outline of the thesis

This thesis was structured in eight chapters that cover the research aims defined above.

The current chapter (**Chapter 1**) presents the contextualization and motivation, research goals and the outline and outputs of this thesis.

Chapter 2 provides a compressive review of biodiesel production, with main focus on microbial lipids potential. Furthermore, the chapter also addresses the biotechnological applications of yeast *Y. lipolytica*, namely for microbial lipids production from VFAs and crude glycerol.

From Chapter 3 to 7 the different sections of experimental results are present. Each chapter contains a brief introduction, material and methods, results and discussion and conclusions.

In **Chapter 3**, the ability of *Y. lipolytica* strains (W29 and NCYC 2904) to grow and accumulate lipids intracellularly using synthetic medium containing VFAs as carbon sources was evaluated in batch cultures. Important factors affecting the bioconversion of VFAs by *Y. lipolytica* W29 and NCYC 2904 for growth and lipids accumulation were studied as an attempt to increase lipids productivity, namely the addition of co-substrates (glucose or glycerol) and operation mode. Furthermore, the effect of oxygen volumetric mass transfer (k_La) on microbial lipids production by *Y. lipolytica* strains from VFAs was assessed in a lab-scale stirred bioreactor.

In **Chapter 4**, it was explored the potential of using *Y. lipolytica* W29 as a biorefinery platform to produce microbial lipids from high-content VFAs media, in two-stage batch cultures performed in Erlenmeyer flasks. Moreover, the estimated fuel properties of biodiesel produced from *Y. lipolytica* W29 lipids were addressed and compared with the biodiesel produced from vegetable oils and international biodiesel standards.

In **Chapter 5**, it was explored the potential of using *Y. lipolytica* NCYC 2904 as a biorefinery platform to produce microbial lipids from high-content VFAs media, in batch and two-stage batch cultures carried out in a lab-scale stirred bioreactor. In addition, the effect of dissolved oxygen concentration during lipogenic phase in VFAs was assessed. The fuel properties of biodiesel produced from *Y. lipolytica* NCYC 2904 lipids were estimated to assess the potential of this biodiesel to replace conventional fuels.

Chapter 6 presents the feasibility of using VFAs produced during the acidogenesis of food waste as the carbon source for *Yarrowia lipolytica* (W29 and NCYC 2904) growth and lipids production. Aerobic batch cultures of *Y. lipolytica* were performed in VFAs-rich fermented medium and crude glycerol as co-substrate. A two-stage batch culture using a VFAs-rich fermented medium for cell proliferation and crude glycerol for lipid synthesis was carried out in a lab-scale stirred bioreactor as an attempt to boost biomass

and microbial lipids production. The integration of an anaerobic and aerobic process generates a value chain from wastes and by-products to microbial lipids production.

Chapter 7 presents the potential of *Y. lipolytica* to convert low-cost substrates into feedstocks for high-quality biodiesel production, according to the biorefinery and bioeconomy circular guidelines. Microbial lipids production by *Y. lipolytica* NCYC 2904 from crude glycerol and VFAs was studied in a lab-scale stirred bioreactor using fed-batch and two-stage batch operation modes.

Chapter 8 refers to the general conclusions obtained in this thesis, and suggestions for following work.

1.3. Outputs of the thesis

According to the 2nd paragraph of article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed below.

- **Peer reviewed journal articles:**

Pereira, A. S., Miranda, S. M., Lopes, M., Belo, I. (2021) Factors affecting microbial lipids production by *Yarrowia lipolytica* strains from volatile fatty acids: Effect of co-substrates, operation mode and oxygen, Journal of Biotechnology. 331: 37-47. https://doi.org/10.1016/j.jbiotec.2021.02.014

Lopes, M., Miranda, S. M., Costa, A. R., **Pereira, A. S.**, Belo, I. (2022) *Yarrowia lipolytica* as a biorefinery platform for effluents and solid wastes valorization - challenges and opportunities. Critical Reviews in Biotechnology 42 (2): 163-183. https://doi.org/10.1080/07388551.2021.1931016

Pereira, A. S., Lopes, M.; Miranda, S. M.; Belo, I. (2022) Bio-oil production for biodiesel industry by *Yarrowia lipolytica* from volatile fatty acids in two-stage batch culture. Applied Microbiology and Biotechnology, 106 (8): 2869-2881. https://doi.org/10.1007/s00253-022-11900-7

Pereira, A. S., Belo, I., Lopes, M. (2022) Enhancing microbial lipids synthesis for biodiesel production by *Y. lipolytica* W29 from volatile fatty acids: two-stage batch strategies. Applied Sciences, 12 (17): 8614. https://doi.org/10.3390/app12178614

Pereira, A. S., Lopes, M, Duarte, M.S., Alves, M.M., Belo, I. (2023) Integrated bioprocess of microbial lipids production in *Yarrowia lipolytica* using food-waste derived volatile fatty acids. Renewable Energy, 202: 1470-1478. https://doi.org/10.1016/j.renene.2022.12.012

Pereira, A. S., Lopes, M., Belo, I. (2023) From crude glycerol and volatile fatty acids to biodiesel and other bioproducts using *Yarrowia lipolytica* NCYC 2904 as a cell factory. Sustainable Energy and Fuels, 7: 4687-4696. https://doi.org/10.1039/D3SE00682D

- **Flash poster presentation**

Pereira, A. S., Lopes, M., Belo, I. High-quality biodiesel production from *Yarrowia lipolytica* NCYC 2904 bio-oil. Biotechnology for a Circular Bioeconomy (AFOB-EFB Virtual Conference), 2023

Pereira, A. S., Lopes, M., Belo, I. Volatile fatty acids as sustainable carbon source for microbial lipids production by *Yarrowia lipolytica*. 2nd Chemical and Biological Engineering Doctoral Symposium. Centre of Biological Engineering, Campus Gualtar, Braga, Portugal, 2021.

- **Oral communications**

Pereira, A. S., Miranda, S. M., Belo, I., Lopes, M. *Yarrowia lipolytica* is a promising oleaginous yeast for bio-oils production from volatile fatty acids. European Biotechnology Congress. Prague, Czech Republic, 2022.

Pereira, A. S., Miranda, S. M., Lopes, M., Belo, I. Bioconversion of volatile fatty acids into microbial lipids by *Yarrowia lipolytica*. 4th Iberoamerican Congress on Biorefineries. Jaén, Spain, 2018.

- **Proceedings**

Pereira, A. S., Miranda, S. M., Lopes, M., Belo, I. Bioconversion of volatile fatty acids into microbial lipids by *Yarrowia lipolytica*. Proceedings of 4th Iberoamerican Congress on Biorefineries. Jaén, Spain, 26-27 October, 2018.

CHAPTER 2

Literature review

2.1. Biodiesel: an alternative substitute to fossil fuels

The global increase in energy consumption, the depletion of fossil fuel reserves, and environmental concerns promoted the development of fuels from renewable and environmentally friendly energy sources. EU remains highly dependent on fossil fuels, which accounted for 70 % of available energy in 2021. Nevertheless, a reduction of dependence on fossil fuels have been decreased over the last decades (Eurostat 2023c). Consequently, an increase in energy consumption from renewable energy has been observed, representing 21.8 % of energy consumed in 2021 (Eurostat 2023d). This value is still below to target set for the EU by 2030 (42.5 %) (European Commission 2023a). Therefore, the development and implementation of alternative and efficient energy sources has been promoted in order to increase the capacity of renewable energy (Bórawski et al. 2019).

Biodiesel is one of the most attractive alternatives for the replacement of conventional fossil fuels due to its biodegradable, non-toxic- and energy-efficient properties (Singh et al. 2020). In the EU, biodiesel production reached 43.2 billion liters and it is expected to reach 53.2 billion by 2026 (IEA 2021). Conventionally, biodiesel is produced by transesterification of lipids extracted from a variety of feedstock, mainly plant/vegetable oils, but these are limited by land availability, influenced by the climatic conditions and competition used for food purposes (Szczepańska et al. 2021; OECD 2022). Furthermore, the main obstacle to the commercialization of biodiesel is its production costs, 1.5 times higher than those of petroleum diesel. About 75 % of the total cost of biodiesel corresponds to prices of edible and non-edible vegetable oils (Gaeta-Bernardi and Parente 2016; Gujjala et al. 2019). Therefore, sustainable and cost-effective feedstocks are needed to overcome these limitations. A promising alternative source for biodiesel production is microbial lipids, whose fatty acids composition is similar to vegetable oils commonly used in biodiesel production. Moreover, microbial lipids production has several advantages comparatively to vegetable oils, such as independence of climatic conditions, seasons and not competition with food crops for land, shorter cultivation period, lipids productivity, and lower surface requirements (Gujjala et al. 2019; Llamas et al. 2020b).

2.2. Microbial lipids

Microbial lipids, also called single cell oils (SCO), microbial oils, or bio-oils, are produced by oleaginous microorganisms, including yeast, fungi, bacteria, and algae that can accumulate high content of lipids (> 20 % on the cell dry weight) in an intracellular particle called lipid body (LB). LBs are formed by neutral lipids, mainly TAGs and steryl esters. The lipid content and long chain fatty acids (LCFA) profile differs between species, however are mainly composed of myristic (C14:0), palmitic (C16:0), palmitoleic

(C16:1), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids (Carsanba et al. 2018; Patel et al. 2020; Bao et al. 2021).

Microbial lipids of some oleaginous microorganisms are valuable sources of polyunsaturated fatty acid (PUFA) that can be used as food supplements or animal feed. Linoleic acid is the major PUFA synthesized by wild-type *Y. lipolytica* strains and is the precursor for the synthesis of more highly unsaturated and longer-chained omega-6 family fatty acids. By using genetically modified strains, it has been possible the production of γ-linoleic acid, arachidonic acid, and conjugated linoleic acid (Liu et al. 2021; Cao et al. 2022). Microbial lipids produced by *Mortierella alpina* are also a source of PUFA, namely linoleic and α-linoleic acid (Ferreira et al. 2021). Microbial lipids can also be used as a substitute for cocoa butter, commonly used in the food industry and, particularly, in chocolate manufacturers. Considering the composition of saturated fatty acid in cocoa butter, ranging from 55 % to 67 % (w/w), numerous strategies have been explored to increase saturated fatty acids, such as the growth of yeasts in a medium rich in stearic acid and genetic manipulation of wild species (Papanikolaou and Aggelis, 2011). Moreover, it has been highlighted the importance of microbial lipids as promising raw-material for biodiesel production since their composition can be similar to that of common vegetable oils. Several studies reported the production of microbial lipids as feedstock for biodiesel, where fatty acids obtained were mostly unsaturated, which are highly suitable for biodiesel production (Carsanba et al. 2020; Bao et al. 2021; Sarantou et al. 2021; Raut et al. 2022; Vasaki et al. 2022). Table 2.1 shows some oleaginous microorganisms that can be used for biodiesel production.

Table 2.1 - Microbial lipids production by oleaginous microorganisms cultivated in several substrates.

Oleaginous Microorganism	Substrate	Lipids content (% w/w)	Reference
Microalgae			
Auxenochlorella protothecoides	Forest biomass hydrolysates.	63 - 66	Patel et al. (2018)
Scenedesmus obliquus	Municipal wastewater	21	Ling et al. (2019)
Chlorella vulgaris	*Dairy wastewater*	36.7	Asadi et al. (2020)
Chlorella orokiniana	*Dairy wastewater*	35	
Tetradesmus bernardii	Glucose	45	Gao et al. (2021)

Table 2.1 - Microbial lipids production by oleaginous microorganisms cultivated in several substrates (continuation).

Oleaginous Microorganism	Substrate	Lipids content (% w/w)	Reference
Yeast			
Cryptococcus curvatus	Waste office paper hydrolysate	22 - 38	Annamalai et al. (2018)
Yarrowia lipolytica	Food waste leachate	49	Johnravindar et al. (2018)
Rhodosporidiobolus fluvialis	Surgacane hydrolysate and crude glycerol	61.4	Poontawee and Limtong (2020)
Cryptococcus podzolicus	Acetate and glucose	38.8	Qian et al. (2020)
Trichosporon porosum	Acetate and glucose	39.7	
Apiotrichum porosum	VFAs and glucose	36.2	Qian et al. (2021)
Rhodosporidium toruloides	Food waste hydrolysate	36.4	Carmona-Cabello et al. (2021)
Bacteria			
Rhodococcus opacus	Biomass gasification wastewater	54.3	Goswami et al. (2017)
Rhodococcus sp.	Food waste-derived VFAs	69	Bhatia et al. (2019)
Rhodococcus opacus	Waste paper hydrolysate	43	Nair and Sivakumar (2022)

Bacteria are characterized by high cell growth rates under simple cultivation, but the low biomass production and difficulty in extracting lipids make microbial lipids production from bacteria unsustainable. Oleaginous microalgae are promising sources of microbial lipids, however microalgae require light conditions and large surfaces of cultivation and have a longer cultivation period than other oleaginous microorganisms. Oleaginous yeasts stand out for their capability to be cultivated on a wide range of low-cost feedstocks, rapid growth, and ease of scaling up the process, which make them interesting microbial factories for microbial lipids production (Patel et al. 2020; Llamas et al. 2020b; Caporusso et al. 2021).

2.3. Microbial lipids production by *Yarrowia lipolytica*

Among all oleaginous microorganisms, *Y. lipolytica* is one of the most attractive hosts since it is a "generally regarded as safe" (GRAS) organism with several potential applications in metabolite synthesis, functional protein production, and environmental bioremediation (Coelho et al. 2010; Groenewald et al. 2014; Liu et al. 2015).

Yarrowia lipolytica is a eukaryotic microorganism belonging to the Fungi kingdom. Initially, this yeast was denominated *Candida lipolytica*. Later, it was reclassified and renamed as *Endomycopsis lipolytica*, *Saccharomycopsis lipolytica,* and, finally, *Y. lipolytica*. The name "lipolytica" refers to the capacity to hydrolyze lipidic substrates (van der Walt and von Arx 1980). *Yarrowia lipolytica* strains have been isolated from dairy products (cheese, yogurt), meat, soil, and oil-polluted environments or marine and hypersaline environments (Groenewald et al. 2014; Jach and Malm 2022). *Yarrowia lipolytica* has the ability to grow in two different morphological forms, specifically yeast single cells or filamentous hyphae. A variety of environmental conditions can affect the cell morphology, namely physical-chemical factors (pH, temperature, and dissolved oxygen concentration), mechanical factors (air pressure and agitation rate), and nutritional factors (carbon and nitrogen sources, metal ions, among others) (Timoumi et al. 2018).

Yarrowia lipolytica stands out for its ability to assimilate a wide range of substrates in many industrially relevant contexts, including sugars from hydrolysis of lignocellulosic biomass (Vasaki et al. 2022), VFAs from acidogenic fermentation of organic wastes (Gao et al. 2020), glycerol from various industries (including biodiesel manufacturing) (Dobrowolski et al. 2016) and also hydrophobic substrates such as fatty acids derived from animal fat (Lopes et al. 2018), olive mill wastewater (Sarris et al., 2023), and waste cooking oils (Lopes et al. 2019). In addition, *Y. lipolytica* is considered a cell factory for the production of valuable compounds of industrial interest such as extracellular enzymes (e.g., lipase and protease) (Lopes et al. 2019), organic acids (e.g., citric acid and succinic acid) (Ong et al. 2019; Papanikolaou et al. 2020), sugar alcohols (e.g., erythritol, mannitol, and arabitol) (Filippousi et al. 2019; Papanikolaou et al. 2020) and aroma compounds (e.g., γ-decalactone, 2-phenylethanol) (Braga et al. 2015; Braga et al. 2021). As an oleaginous microorganism, *Y. lipolytica* has also the ability to accumulate lipids intracellularly and most of the storage lipids are in the form of triacylglycerols (TAGs). The similarity of fatty acids composition of lipids accumulated by *Y. lipolytica* and the vegetable oils makes *Y. lipolytica* a promising candidate for biodiesel production (Carsanba et al. 2018; Gao et al. 2020; Carsanba et al. 2020).

Microbial lipids are accumulated by *Y. lipolytica* via two different metabolic pathways: (a) *de novo* synthesis, involving the production, in defined conditions, of fatty acid precursors such as acetyl and

malonyl-CoA, and their integration into the lipid biosynthetic pathway from hydrophilic substrates; and (b) the *ex novo* synthesis, involving the incorporation of exogenous fatty acids, oils, and TAGs from the hydrophobic carbon sources (Carsanba et al. 2018). The main metabolic routes of *de novo* and *ex novo* lipids synthesis from hydrophilic and hydrophobic carbon sources described for *Y. lipolytica* are depicted in Figure 2.1. The *de novo* synthesis is induced by the exhaustion or limitation of a primary nutrient from the culture medium, namely nitrogen. When nitrogen becomes unavailable, cell proliferation slows down since it is an essential nutrient for protein and nucleic acid synthesis. However, the cell continues to assimilate the carbon source from the medium, which is now channeled to lipid synthesis. In the case of glucose, it is transported into the cytoplasm and, through glycolysis, are formed glycerol-3-phosphate and pyruvate. Pyruvate enters the mitochondria where it is decarboxylated into acetyl-CoA and then enters the tricarboxylic acid cycle (TCA). When the nitrogen is limited, the concentration of intracellular adenosine monophosphate (AMP) decreases, and subsequently isocitrate dehydrogenase (IDH) is inhibited, downregulating the TCA cycle. This leads to the accumulation of citrate inside the mitochondria and when the citrate concentration reaches a critical value, citrate is transported into the cytosol. Then, the citrate is cleaved by ATP-citrate lyase (ACL) into acetyl-CoA and oxaloacetate, an enzymatic activity crucial for fatty acid biosynthesis. The conversion of acetyl-CoA to malonyl-CoA by acyl-CoA carboxylase (ACC) is the principal source of carbon atoms for *de novo* fatty acid synthesis. The enzymatic complex fatty acid synthase produces acyl-CoA using acetyl-CoA as an initiation molecule and malonyl-CoA as an elongation unit. Most of the released acyl-CoA corresponds to chain lengths of 16 and 18 carbons, which are substrates for the enzymes elongases and desaturases. Elongases are responsible for chain elongation, producing long-chain fatty acids (LCFA). Desaturases, located in the endoplasmic reticulum, are responsible for first desaturation to produce palmitoleic (C16:1) or oleic (18:1) acids and for a second double bond to produce linoleic acid (C18:2). The TAGs synthesis commonly follows the Kennedy pathway, where three fatty acids are condensed with one glycerol-3-phosphate (G3P). This pathway consists of acylation of G3P to lysophosphatidic acid (LPA) by G3P acyltransferase (SCT1). Subsequently, LPA is acylated to phosphatidic acid (PA) by LPA acyltransferase (SLC1) and then phosphorylated to diacylglycerol (DAG) by PA phosphatase (PAP). Finally, the enzymes DAG acyltransferases (DGA1 and DGA2) incorporate the third acyl-CoA onto the DAG, forming TAGs (Beopoulos et al. 2009; Ledesma-Amaro and Nicaud 2016a; Zeng et al. 2018).

Glycerol is incorporated into yeast and is directly phosphorylated by glycerol kinase (GUT1) into G3P, and then is acylated into LPA to TAG synthesis. The G3P produced is also converted to dihydroxyacetone

phosphate (DHAP) by FAD^+ - dependent G3P dehydrogenase (GUT2) and this DHAP may enter glycolysis or gluconeogenesis (Zeng et al. 2018).

VFAs are incorporated by the yeast cells through a proton symport and converted into acetyl-CoA, a central intermediate in lipid synthesis. In the case of acetate, this is converted directly into acetyl-CoA by acetyl-CoA synthase (ACS). Propionate is converted to propionyl-CoA, which enters the methyl citrate cycle to produce pyruvate as its final. The pyruvate is then decarboxylated to acetyl-CoA. Butyrate is converted to butyryl-CoA and then to acetyl-CoA via β-oxidation. The acetyl-CoA formed can be directly incorporated into lipids or transported into the mitochondria and enter in the TCA cycle for energy production (Vajpeyi and Chandran 2015; Liu et al. 2016).

In the presence of lipidic substrates, *Y. lipolytica* secretes extracellular lipases to hydrolyse fats and oils into free fatty acids (FFA) outside the cell, which will be incorporated into the cell. In the cytosol, these FFA are activated by fatty acyl-CoA synthetase (FAA1) to produce acyl-CoA, which can be used for lipid synthesis. However, these FFA can also enter directly the peroxisome to produce acetyl-CoA via *β*-oxidation and are used as an energy source by the cell. The hydrophobic substrates in the culture medium are incorporated into the cell through the modification of surface hydrophobicity, or by the presence of protrusions that increases the contact surface between the yeast and hydrophobic substrates (Ledesma-Amaro and Nicaud 2016b; Soong et al. 2023).

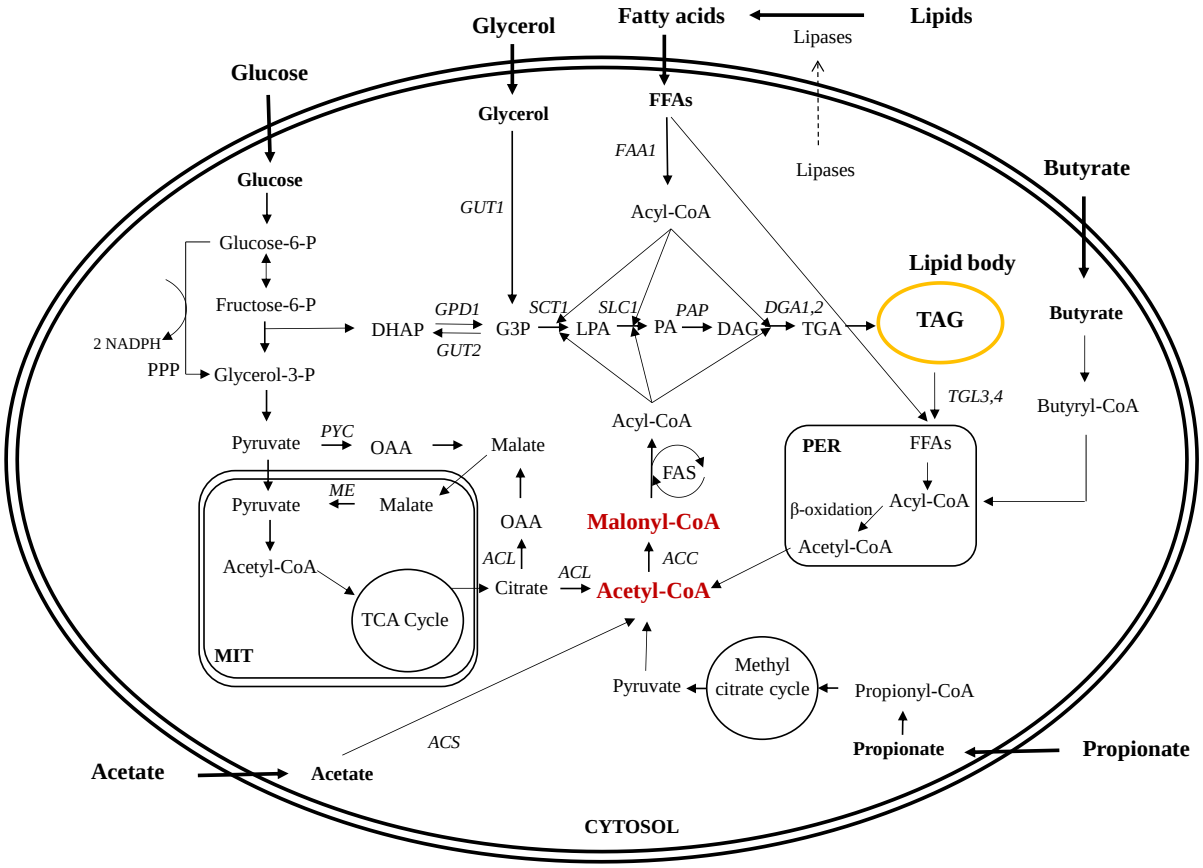

Figure 2.1 - Schematic representation of the metabolic pathways of lipids production from glucose, glycerol, fatty acids, and volatile fatty acids (acetate, propionate, and butyrate). MIT, mitochondria; PER, peroxisome; LB, lipid body; TCA, tricarboxylic acid cycle; PPP, pentose phosphate pathway; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate, LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TGA, triacylglycerol; FFAs, free fatty acids; PYC, pyruvate carboxylase, ME, malic enzyme; ACL, ATP-citrate lyase; ACL, acetyl-CoA carboxylase; ACC, acyl-CoA carboxylase; FAS, fatty acid synthase; GUT1, glycerol kinase; GPD1, NAD+ - dependent G3P dehydrogenase; GUT2, FAD+ - dependent G3P dehydrogenase; SCT1, G3P acyltransferase; SLC1, LPA acyltransferase; PAP, PA phosphohydrolase; DGA1,2, DAG acyltransferases I and II; TGL3,4, TAG lipases 3 and 4.

2.3.1. Factors affecting microbial lipids production

Lipids accumulation by *Y. lipolytica* can be affected by some factors, namely the carbon/nitrogen (C/N) ratio, carbon and nitrogen sources, dissolved oxygen, and mode of operation. Several carbon sources have been used by *Y. lipolytica* for microbial lipids production. Table 2.2 describes some studies regarding microbial lipids production by *Y. lipolytica* strains from different substrates.

Table 2.2 - Microbial lipids production by *Y. lipolytica* strains from different substrates.

Strain	Substrate	Lipids content (% w/w)	Lipids concentration (g/L)	Reference
W29	Glucose	35	1.3	Carsanba et al. (2020)
	Waste cooking oil	48	3.0	Lopes et al. (2019)
	Pork lard	58	5.0	Lopes et al. (2018)
CICC 31596	Glucose	37	0.9	Gao et al. (2017)
MTCC 9519	Lignocellulosic biomass	59	6.7	Satya Sagar et al. (2023)
DSM 8218	Wheat straw hydrolysate	8- 13	3.8 – 5.3	Caporusso et al., (2023)
NCIM 3589	Waste cooking oil	25 - 30	1.5 – 2.5	Raut et al. (2022)
KKP 379	Waste fish oil	23	4.6	Fabiszewska et al. (2021)

Yarrowia lipolytica has the extraordinary ability to assimilate a wide range of substrates for lipid synthesis. Carsanba et al. (2020) investigated microbial lipids synthesis by different *Y. lipolytica* strains from glucose under nitrogen-limited conditions, demonstrating that *Y. lipolytica* W29 accumulated 35 % of lipids in dry cell weight. Lignocellulosic biomass is considered an alternative carbon source for lipids production by *Y. lipolytica*, since the hydrolysis of lignocellulosic biomass originates fermentable sugars, such as glucose, xylose, mannose, and arabinose. For example, Satya Sagar et al. (2023) reported that *Y. lipolytica* can produce lipids up to 6.7 $g \cdot L^{-1}$ from lignocellulosic biomass. Moreover, several studies showed that *Y. lipolytica* can also use hydrophobic substrates as carbon sources for lipid production. In waste cooking oil (WCO)-based media, *Y. lipolytica* W29 and *Y. lipolytica* NCIM 3589 produced about 3 $g \cdot L^{-1}$ of microbial lipids (Lopes et al. 2019; Raut et al. 2022). *Yarrowia lipolytica* W29 has also the ability to accumulate a great content of lipids (5.0 $g \cdot L^{-1}$) from pork lard, used as a model of animal fat (Lopes et al. 2018). Fabiszewska et al. (2021) also concluded that *Y. lipolytica* has the ability to grow and accumulate lipids from waste fish oil.

In addition to carbon sources, nitrogen is one of the most important components of microbial media since it is required for important metabolic processes such as nucleotide and protein biosynthesis. The nitrogen source can affect lipids accumulation and its limitation in the medium is important (Carsanba et al. 2018; Bao et al. 2021). Kolouchová et al. (2016) studied the effect of nitrogen source (ammonium chloride) on

lipid production by *Y. lipolytica* CCY 29-26-36 in a glucose medium and concluded that nitrogen limitation favours lipids production. Urea and ammonium sulfate are other nitrogen sources tested for lipids production by *Y. lipolytica* (Brabender et al. 2018; Wierzchowska et al. 2021). Microbial lipids production can also be promoted by phosphorus limitation and the combination of both phosphorus and nitrogen sources was used to optimise lipids production by *Y. lipolytica* CCY 29-26-36 and *Y. lipolytica* KKP 379 from glucose medium and waste oil, respectively (Kolouchová et al. 2016; Wierzchowska et al. 2021).

For *de novo* lipids synthesis, the C/N ratio is the most important factor that affects lipids accumulation by *Y. lipolytica*. The excess of carbon and nitrogen depletion leads to high lipids content, as a consequence of a stress response or of an adaptation to a nutrient medium limitation (Beopoulos et al. 2009). Microbial lipids accumulation by *Y. lipolytica* strains is induced at a C/N ratio above 20 (Carsanba et al. 2018). Fontanille et al. (2012) reported that *Y. lipolytica* MUCL 28849 accumulated up to 37 % of lipids from glucose and glycerol, using a C/N ratio of 62. Dobrowolski et al. (2016) observed higher lipids production by *Y. lipolytica* A101 at C/N ratios between 75 to 150 in glycerol-based medium. C/N ratios ranging from 25 to 150 were investigated in *Y. lipolytica* SKY 7 cultures and C/N 100 resulted in the highest lipids production from crude glycerol (Kuttiraja et al. 2016). Moreover, Morales-Palomo et al. (2022) studied the effect of C/N (90 to 200) in lipids production by *Y. lipolytica* ACA DC 50109 from VFAs and it is observed the effect of C/N depends on VFAs profile in the media. A VFAs mixture with a high proportion of short-chain fatty acids combined with high C/N ratio led to higher lipids synthesis by yeast.

Another important factor for lipids production by *Y. lipolytica* is medium pH since this parameter affects the surface properties of the cell membrane, consequently, the carbon source assimilation. According to several studies, the lipids accumulation is favored by a slightly acidic pH (5 - 6.5). Lopes et al. (2018) and Lopes et al. (2019) studied the effect of individual factors and showed a slight decrease in the lipids content of *Y. lipolytica* W29 when varying pH from 5.6 to 7.2 in pork lard-based and WCO-based media. For lipids production by *Y. lipolytica* MUCL 28849, the pH was controlled at 5.6 (Fontanille et al. 2012). The lipid production by *Y. lipolytica* SKY7 in a crude glycerol-based medium was 15 % improved with pH control at 6.5, compared to uncontrolled pH cultures (Kuttiraja et al. 2018). Surprisingly, Zhang et al. (2019a) reported a 50 % decrease in the lipids content of *Y. lipolytica* W29 when the pH of glucose media increased from 2 to 6.

Temperature can also influence the lipids production by *Y. lipolytica*. Papanikolaou et al. (2002) demonstrated that higher amounts of lipids were synthesized at temperatures 28 °C - 30 °C. Gao et al. (2017) showed that *Y. lipolytica* CICC 31596 achieved similar lipids content at temperatures of 28 °C and

38 °C. However, temperature affected the fatty acids profile, where higher temperatures (38 °C) led to a decrease in the content of unsaturated fatty acids comparatively to the cultures carried out at 28 °C.

Dissolved oxygen concentration in *Y. lipolytica* cultures also affects lipid production (Carsanba et al. 2018; Magdouli et al. 2018). Low levels of dissolved oxygen have been described as important for lipids production. Papanikolaou et al. (2007) reported that *Y. lipolytica* ACA DC 50109 accumulated low quantities of microbial lipids (7 % - 16 %, w/w) from an industrial derivative of tallow at high aeration and agitation conditions, which correspond to a high oxygen concentration in the medium. Lopes et al. (2019) reported that *Y. lipolytica* W29 accumulated high lipids content from WCO (48 %, w/w) at low dissolved oxygen concentration. Lopes et al. (2018) evaluated the effect of oxygen transfer rate (OTR) on lipid accumulation by *Y. lipolytica* W29 from pork lard and reported that no improvement of lipids content was achieved by increasing OTR from 122 $mg \cdot L^{-1} \cdot h^{-1}$ to 605 $mg \cdot L^{-1} \cdot h^{-1}$. Studies conducted by Magdouli et al. (2018) also showed that *Y. lipolytica* SM7 accumulated high lipids content in crude glycerol medium with low dissolved oxygen levels. By contrast, Bellou et al. (2014) observed that *Y. lipolytica* ACA DC 50109 accumulated high lipids content from glycerol at high dissolved oxygen levels.

The design of bioprocesses for microbial lipids production should be conceived taking into account the factors that affect lipids production. Different modes of operation of *Y. lipolytica* cultures have been carried out such as batch and fed-batch. The fed-batch mode of cultivation has been considered effective in increasing both the cell density and intracellular lipids of oleaginous microorganisms (Xu et al. 2018; Bao et al. 2021). In a study conducted by Xu et al. (2018), approximately a 3-fold enhancement in lipids concentration was attained in fed-batch mode compared to *Y. lipolytica* batch cultures. Furthermore, a two-stage cultivation mode has been adopted to enhance lipids production by combinations of two modes of operation (Karamerou and Webb 2019; Bao et al. 2021). Fontanille et al. (2012) used a two-stage fed-batch system to achieve high biomass and subsequent conversion of VFAs to microbial lipids by *Y. lipolytica*. Initially, it was obtained high cell density from glucose or glycerol and after the exhaustion of the carbon source, VFAs and ammonium sulfate were added to the medium and the C/N ratio was maintained close to 50. However, several studies on lipids production are performed in batch cultures. For example, crude glycerol from various industries was evaluated to produce lipids by *Y. lipolytica* A101 in batch cultures, performed in shake-flasks and a bioreactor (Dobrowolski et al. 2016). In Lopes et al. (2018), the effect of pH, substrate concentration, arabic gum concentration, and OTR on lipids accumulation by *Y. lipolytica* was studied in batch cultures (shake-flasks and bioreactor). Magdouli et al. (2018) investigated the effect of oxygen levels on cellular growth and lipids production in *Y. lipolytica*

batch cultures. Morales-Palomo et al. (2022) also performed batch cultures to evaluate the effect of VFAs concentration and C/N ratio on lipids production by *Y. lipolytica*.

Over the years, extensive efforts have been made to increase the lipids content of *Y. lipolytica*. In consequence, bioengineered strains were constructed to accumulate higher lipids content, by increasing the expression of lipid synthesis pathways and synthetic precursors, disrupting competing pathways, such as β-oxidation, and eliminating inhibiting factors. Table 2.3 summarizes the impact of some genetic modifications performed in *Y. lipolytica* to enhance intracellular lipids biosynthesis.

Table 2.3 - Genetic modifications performed in *Y. lipolytica* to enhance lipids production.

Gene	Main Results	Reference
HXP1 overexpression	Lipids content increasing (23 % - 55 %)	Lazar et al. (2014)
GDP1 overexpression	Lipids content increasing (1.5-fold)	Dulermo and Nicaud (2011)
GDP1 overexpression, *GUT2* deletion	Lipids content increasing (5.6-fold)	
TGL3, *TGL4* repression	Lipids content increasing	
POX1-6 deletion, *GPD1* overexpression	Lipids content increased by 70 %	
ACL overexpression	Lipids content increased from 7 % to 23%	Zeng et al. (2018)
Snif deletion	Lipids content increased 2.6-fold	
MIG1 deletion	Lipids content increased from 36 % to 49 %	
ACC1, *DGA* overexpression	Lipids content increased up to 41 %	
DGA1&*2* overexpression, *TGL3* delection	Lipids content increased by 77 %	Friedlander et al. (2016)
DGA2 overexpression	Lipids titer, content, and yield increased by 236 %, 165 %, and 246 %, respectively	Silverman et al. (2016)
SLC1 overexpression	Lipids titer, content, and yield increased by 99 %, 91 %, and 151 %, respectively	

One example of improvement in lipids production is the redirecting carbon flux toward lipids synthesis. A mutant strain of *Y. lipolytica* W29, obtained by modifying hexokinase activity (*HXK1*), which is responsible for the phosphorylation of hexoses, showed a significant increase of lipids synthesis from 1.56 $g \cdot L^{-1}$ to 3.02 $g \cdot L^{-1}$ (Lazar et al. 2014). Lipids accumulation was also enhanced in this yeast by elevating the levels of G3P in a strain overexpressing the *GPD1* gene (Dulermo and Nicaud 2011). Lipids overproduction by

oleaginous organisms requires a continuous co-factor NADPH supply, which comes mainly from the pentose phosphate pathway (PPP), where up-regulation of glucose-6-phosphate dehydrogenase (*GPD1*) and 6-phosphoglu-conolactonase (*SOL3*) can increase NADPH supply (Silverman et al. 2016). ATP-citrate lyase (encoded by *ACL* gene) is an enzymatic system that is crucial for lipids synthesis and, when the engineered strain over-expressed *ACL*, a high amount of acetyl-CoA is generated, increasing the lipids production from 7 % to 23 % (w/w). In addition, the simultaneous overexpression of *AAC1* and *DGA* genes resulted in an increase of lipids content up to 41 % (Zeng et al. 2018). The overexpression of *DGA1* and *DGA2* genes and the disruption of the native *TGL3* gene (encoding intracellular triacylglycerol lipase), led to a higher lipids content of 77 % with a yield of 0.21 $g \cdot g^{-1}$ from glucose (Friedlander et al. 2016). In the modified strain overexpressing the *DGA2* gene, an increase of lipids titer, content, and yield of 236 %, 165 %, and 246 %, respectively, was obtained comparatively to the wild strain. In the same way, the overexpression of the *SLC1* gene led to a 99 %, 91 %, and 151 % increase in lipids titer, content, and yield, respectively (Silverman et al. 2016).

The downregulation and disruption of the β-oxidation pathway can reduce or prevent the degradation of accumulated TAGs. For example, the overexpression of *GPD1* and simultaneous inactivation of *GUT2* resulted in a 5.6-fold improvement in G3P concentration and, consequently, in the increase of TAGs accumulation. The repression of *TGL3* and *TGL4* genes, both responsible for TAGs degradation, improved the lipids accumulation by *Y. lipolytica*. The β-oxidation pathway can be damaged by inactivation of *POX* genes, which encode different acyl-coenzymes. Inactivation of *POX1-6* genes combined with *GPD1* overexpression led to a 70 % increase in lipids content (Dulermo and Nicaud 2011).

The cellular metabolism regulators play a significant role in lipids production. For example, *Y. lipolytica* with a deletion of *the Snif1* gene accumulated 2.6-times higher lipids than the wild type. The *Snif1* gene is one of the major negative regulators of lipids production in yeast. A similar observation was found for strains with *MIG1* gene deletion (*MIG1* is the major constituent of the glucose repression pathway in *Y. lipolytica*), enhancing lipids content from 36 % to 49 % (w/w) (Zeng et al. 2018).

2.4. Low-cost feedstocks for microbial lipids production

The use of low-cost carbon sources is essential for the economic viability of the process. In fact, *Y. lipolytica* has the feasibility to grow and produce lipids from wastes and by-products of the agro-industrial sector, such as waste cooking oils and lignocellulosic biomass, making the process of microbial lipids more viable (Lopes et al. 2019; Caporusso et al. 2023). However, considerable efforts have been directed to find new alternative carbon sources to reduce the cost of microbial lipids production, including VFAs

(products from acidogenic fermentation of organic wastes) and crude glycerol (a by-product of biodiesel production).

2.4.1. Volatile fatty acids (VFAs)

VFAs, short-chain fatty acids with two to six carbon atoms produced through anaerobic digestion (AD), are considered building blocks for chemicals and biofuels, which allow the replacement of fossil fuels-based products, favouring the circular economy and environmental sustainability (Atasoy et al. 2018; Llamas et al. 2020b; Agnihotri et al. 2022). Anaerobic digestion is an effective, eco-friendly, and sustainable technology for organic waste treatment and valorization by the conversion of the organic matter present in waste into added-value products, such as methane, hydrogen, and VFAs (Duarte et al. 2021; Lytras et al. 2021). This process consists of a complex microbial process where, in the absence of oxygen, consortia of microorganisms use biodegradable waste for energy production by breaking down organic matter (Náthia-Neves et al. 2018; Lytras et al. 2021). For example, food waste, rich in carbohydrates, proteins, and lipids, is firstly hydrolyzed into dissolved simple compounds (e.g., sugars, amino acids, and fatty acids) by enzymes excreted by hydrolytic microorganisms (hydrolysis step). The action of acidogenic fermentative microorganisms involves the conversion of soluble compounds (previously obtained by hydrolysis) into VFAs and alcohols (acidogenesis step). In the acetogenic step, the acidogenesis products are further digested and converted into acetic acid, hydrogen, and carbon dioxide by acetogenesis microorganisms. In the final step (methanogenesis), methane and carbon dioxide are derived from acetogenesis products by methanogenic bacteria. Thus, AD allows the treatment of food waste while producing biogas (a mixture of methane and carbon dioxide), which can be used as an energy source in a variety of sectors including transport, industry, power, and heating. Moreover, AD also results in a digestate rich in nutrients (mineral nitrogen, phosphorus, and organic matter) that can be used as an organic fertilizer in agriculture (Náthia-Neves et al. 2018; Chew et al. 2021; Lytras et al. 2021). More specifically, VFAs are produced after the hydrolysis and acidogenesis steps of the AD process, and their production is enhanced through the inhibition of the methanogenesis step which hinders VFAs from being consumed and converted to methane. A mixture of VFAs is obtained from anaerobic fermentation whose composition and concentration depend on operational conditions, substrate, and microbial population (Moretto et al. 2019; Moza et al. 2022; Dahiya et al. 2023). Acetic, propionic, butyric, iso-butyric, iso-valeric, valeric, and caproic acids are the principal VFAs produced during the acidogenesis of food waste (Table 2.4).

Table 2.4 - Composition of VFAs produced by acidogenesis from different organic waste sources.

Substrate	Total VFAs ($g \cdot L^{-1}$)	Acetic acid ($g \cdot L^{-1}$)	Propionic acid ($g \cdot L^{-1}$)	Butyric acid ($g \cdot L^{-1}$)	Isobutyric acid ($g \cdot L^{-1}$)	Valeric acid ($g \cdot L^{-1}$)	Isovaleric acid ($g \cdot L^{-1}$)	Caproic acid ($g \cdot L^{-1}$)	Reference
Food waste	40.19	5.63	3.62	20.10	1.21	0.40	0.80	-	Yasser Farouk et al. (2023)
Food waste	19.66	11.07	3.56	3.44	0.44	0.66	0.53	-	Yang et al. (2022)
Food waste Thermal-hydrolyzed sewage sludge	10.67	8.62	0.15	1.76	-	0.14	-	-	Gong et al. (2021)
Food waste Crude glycerol Brine wastewater	15.62	5.84	5.33	3.06	-	0.75	-	0.64	Duarte et al., 2021
Food waste	35.35	16.11	6.69	10.75	0.35	0.83	0.62	-	Gao et al. (2020)
Fruit and vegetable waste	22.18	5.34	1.02	14.23	0.21	0.92	0.46	-	
Food waste	27.4	5.07	2.37	13.03	< 4 %	< 4%	< 4%	4.30	Wainaina et al. (2020)
Food waste	6.12	4.04	0.86	1.22	-	-	-	-	Chatterjee and Mohan (2020)
Food waste	25.94	9.35	8.61	4.51	-	2.84	-	0.63	Moretto et al., 2019
Food waste	41.06	19.54	3.65	10.84	-	-	-		(Liu et al. 2017b)
Food waste	28.94	13.10	5.24	8.43	0.41	0.79	0.97	-	Gao et al. (2017)

Waste-derived VFAs have multiple novel applications in biological processes including the production of bioplastics, bioenergy generation, and biological nutrient removal (Moza et al. 2022; Agnihotri et al. 2022). VFAs play an important role in environmental remediation processes through their action in removing biological nutrients such as nitrogen and phosphorus in wastewater (Tang et al. 2019). In addition, waste-derived VFAs can be used as a carbon source by microorganisms (bacteria or microbial mixed cultures) for the production of biodegradable polymers, polyhydroxyalkanoates, under nutrient-limited conditions of nitrogen, phosphorus, magnesium, or oxygen (Vu et al. 2021). Waste-derived VFAs are considered promising low-cost feedstocks in the cultivation of oleaginous microorganisms for microbial lipids production (Bhatia et al., 2019b; Morales-Palomo et al., 2022; Patel et al., 2021). Notably, *Y. lipolytica* has the capacity to accumulate lipids from VFAs, making it a particularly suitable platform for biodiesel production (Table 2.5).

Table 2.5 - Microbial lipids production by several *Y. lipolytica* strains from VFAs.

Strain	Operation mode	Carbon source	Lipids content (% w/w)	Lipids concentration (g/L)	Reference
MUCL 28849	Two-stage fed-batch	VFAs	25 - 31	0.85 - 1.84	Fontanille et al. (2012)
CCY 29-26-36	Batch (flask)	VFAs	9 - 13	0.31 - 0.56	Kolouchová et al. (2015)
CICC 31596	Batch (flask)	Acetic acid	13 - 32	0.7 - 1.2	Gao et al. (2017)
		Propionic acid	22 - 28	0.6	
		Butyric acid	20 - 29	0.5	
		Mixture VFAs	22	0.7	
		VFAs from AD of food waste	16 - 18	1.7 - 2.0	

Table 2.5 - Microbial lipids production by several *Y. lipolytica* strains from VFAs (continuation).

Strain	Operation mode	Carbon source	Lipids content (% w/w)	Lipids concentration (g/L)	Reference
CICC 31596	Batch (flask)	Mixture of VFAs	19 - 31	3 - 8	Gao et al. (2020)
		VFAs from AD of food waste	15 - 22	1.4 - 3.2	
		VFAs from AD of fruit and vegetable waste	18 - 26	1.2 - 3.1	
ACA DC 50109	Batch (flask)	VFAs from AD of *Chlorella vulgaris* biomass	15 - 20	0.4 - 0.7	Llamas et al. (2020a)
YICC 31,596	Batch (flask)	VFAs from AD of food waste	33	3	(Xu et al. 2022)
ACA DC 50109	Batch (flask)	VFAs from AD of food waste	37	-	(Morales-Palomo et al. 2022)
CECT 1240	Batch (flask)	VFAs derived from oxidised sewage sludge	35	1.1	Moreno et al. (2023)

2.4.2. Crude glycerol

Crude glycerol is also of particular interest as a low-cost feedstock for the production of microbial lipids (Dobrowolski et al. 2016; Sarantou et al. 2021). Crude glycerol is generated from several industries, such as soap, fatty acid, and fatty esters, however, its major source is the biodiesel industry. In the case of biodiesel production, crude glycerol represents approximately 10 % of total biodiesel (Monteiro et al. 2018; Kumar et al. 2019). Considering the increase in demand for biodiesel production, high amounts of crude glycerol are expected to be produced, whereby crude glycerol has received much attention as a source of cheap raw-material for the production of several added-value compounds, including microbial lipids (Filippousi et al. 2019; Papanikolaou et al. 2020; Sarantou et al. 2021). Crude glycerol is part of the wide range of carbon sources utilized by *Y. lipolytica* for microbial lipids production (Table 2.6)

Table 2.6 - Microbial lipids production by several *Y. lipolytica* strains from crude glycerol.

Strain	Operation mode	Lipids content (% w/w)	Lipids concentration (g/L)	Reference
A101	Batch (flask)	25	1.7	Dobrowolski et al. (2016)
SKY7	Batch	46	7.8	Kuttiraja et al. (2018)
SMT	Batch	48	8	Magdouli et al. (2018)
FMCC Y73, FMCC Y75, FMCC Y74	Batch (flask)	3 - 19	0.4 – 1.2	Filippousi et al. (2019)
LMBF Y-46	Batch (flask)	19	1.3	Diamantopoulou et al. (2020)
ACA-DC 5033 LFMB Y19	Batch (flask)	48 8	2.3 0.9	Sarantou et al. (2021)
SKY7	Fed-batch	5 - 50	5 - 15	Kumar et al. (2021)

Yarrowia lipolytica stands out for its ability to assimilate several cost-effective carbon sources for lipids production with a composition similar to vegetable oils commonly used for biodiesel production (Lopes et al. 2019; Vasaki et al. 2022). Thus, besides the already reported work on *Y. lipolytica* biotechnological applications, further work on the improvement and validation of bioprocesses using *Y. lipolytica* as a biorefinery platform for biodiesel production from low-cost feedstocks, is still of utmost importance since it may contribute to the reduction of the price of microbial lipids, allowing to boost their application as an alternative to vegetable oils.

CHAPTER 3

Factors affecting microbial lipids production by *Yarrowia lipolytica* strains from volatile fatty acids

This chapter is based on the following original research article:

Pereira, A. S., Miranda, S. M., Lopes, M., Belo, I. (2021) Factors affecting microbial lipids production by *Yarrowia lipolytica* strains from volatile fatty acids: Effect of co-substrates, operation mode and oxygen. Journal of Biotechnology, 331: 37-47. https://doi.org/10.1016/j.jbiotec.2021.02.014

3.1. Introduction

The growth of worldwide population and consequent human activity lead to a continuous increase in residues generation. In 2018, 220 million tons of municipal wastes (about 492 kg per capita) were generated in the European Union (PORTDATA 2020a) and only 47 % was recycled (PORTDATA 2020b). In some countries, more than 70 % of municipal wastes were incinerated or disposed of in landfill with negative environmental impacts, including water and soil pollution due to the leachate and greenhouse gases emission resulting from decomposition of biodegradable waste (Scherhaufer et al. 2018). Anaerobic digestion (AD) is an efficient technology for the treatment of organic fraction of municipal wastes with great potential to produce biofuels (biogas, biohydrogen) and chemicals, such as volatile fatty acids (Gameiro et al. 2016; Strazzera et al. 2018). Volatile fatty acids (VFAs), produced in acidogenesis and acetogenesis steps of AD of organic wastes, are considered important building blocks for chemical industries, since they are precursors of reduced compounds (alcohols, aldehydes and ketones). Moreover, VFAs can be used as substrate for several microbiological processes, as carbon and energy sources for the production of added-value compounds (Lee et al. 2014; Strazzera et al. 2018).

The non-conventional yeast *Yarrowia lipolytica* has the ability to grow and accumulate lipids up to 50 % of its cell dry mass from wastes and by-products of agro-industrial sector (Papanikolaou et al. 2007; Tsigie et al. 2012b; Lopes et al. 2018). Some studies on lipids production by *Y. lipolytica* have been performed with VFAs as sole carbon source, and most of them reported that high VFA concentrations (above 5 $g \cdot L^{-1}$) had an inhibitory effect on yeast growth (Rodrigues and Pais 2000; Fontanille et al. 2012; Kolouchová et al. 2015; Gao et al. 2017). Lipids accumulated by *Y. lipolytica* using VFAs as carbon source have similar fatty acid composition to common vegetable oils, making them a suitable raw material for biodiesel production. Conventionally, biodiesel is produced by transesterification of lipids extracted from a variety of feedstocks, including plant/vegetable oils, but the high cost and limited supply of these raw materials are the major hindrance for commercial production of biodiesel. The cost of these feedstocks corresponds to 75 % of total cost of biodiesel produced from edible and non-edible vegetable oils (Gaeta-Bernardi and Parente 2016). Furthermore, pure vegetable oils are widely employed for food purposes, reducing their availability for biodiesel production. Therefore, an alternative approach for economical and sustainable biodiesel production may be based on the use of microbial lipids produced from organic waste-derived VFAs by oleaginous microorganisms, favoring the circular economy. Thus, this work addresses the factors that influence the growth and microbial lipids production from VFAs by *Y. lipolytica* strains, comparing two strains performance (W29 and NCYC 2904). Batch cultures in VFAs and with glucose or glycerol as co-substrate were performed, as well as two-stage batch cultures (first stage – growth in glucose; second

stage – lipids production in VFAs) in order to improve yeast growth and lipids synthesis. *Y. lipolytica* is a strictly aerobic yeast and the amount of oxygen available is an important parameter affecting yeast metabolism. Besides cellular growth (Lopes et al. 2009b), also the production of lipase (Lopes et al. 2019), critic acid (Ferreira et al. 2016) and γ-decalactone (Braga et al. 2015) by *Y. lipolytica* W29 were improved by the increase of oxygen transfer rate. The accumulation of lipids from lard (Lopes et al., 2018) and waste cooking oils (Lopes et al., 2019) also proved to be affected by dissolved oxygen concentration. Thereby, the effect of oxygen availability on microbial lipids production from VFAs was assessed, for the first time, in a laboratory-scale bioreactor (stirred tank bioreactor).

3.2. Materials and Methods

3.2.1. Yeast strains and pre-inoculum

Yarrowia lipolytica W29 (ATCC 20460) and *Y. lipolytica* NCYC 2904 cells, previously grown in YPD medium (20 $g \cdot L^{-1}$ peptone, 10 $g \cdot L^{-1}$ yeast extract and 20 $g \cdot L^{-1}$ glucose), were maintained in cryo-stocks (800 μL of yeast culture and 200 μL of glycerol 99.5 %) and stored at - 80 °C until used to prepare inoculum cultures. One cryostock was used to inoculate the pre-inoculum (YPD medium), which was incubated at 170 rpm and 27 °C in an orbital incubator. After 15 h of growth, yeast cells were centrifuged and resuspended in the culture medium at an initial biomass concentration of 0.5 $g \cdot L^{-1}$.

3.2.2. Batch cultures

Batch cultures were carried out in 500-mL baffled Erlenmeyer flasks containing 200 mL of VFAs-based medium, composed by 5 $g \cdot L^{-1}$ VFAs (acetate, propionate or butyrate), 0.5 $g \cdot L^{-1}$ yeast extract, 1.7 $g \cdot L^{-1}$ YNB (without amino acids and without ammonium sulfate) and ammonium sulfate in variable amounts according to carbon source in order to obtain an initial C/N ratio of 75. Initial medium pH was adjusted to 6 and batch cultures were carried out at 27 °C and 170 rpm in an orbital incubator.

Additionally, batch cultures with addition of 5 $g \cdot L^{-1}$ glucose to each VFAs-based medium were also carried out to study the effect of the co-substrate on *Y. lipolytica* W29 growth and lipids accumulation. Since the fermentative broth obtained in acidogenesis of organic wastes is composed by various VFAs, the ability of *Y. lipolytica* to assimilate a mixture of VFAs was also evaluated. Thus, batch experiments were performed with *Y. lipolytica* W29 and NCYC 2904 strain growing in a VFAs-based medium composed by mixture of VFAs (2 $g \cdot L^{-1}$ acetate, 2 $g \cdot L^{-1}$ propionate and 2 $g \cdot L^{-1}$ butyrate), 20 $g \cdot L^{-1}$ co-substrate (glucose or

glycerol), 0.5 g·L^{-1} yeast extract, 1.7 g·L^{-1} YNB and ammonium sulfate (the amount varied depending on co-substrate type, to obtain a C/N ratio of 75).

3.2.3. Two-stage batch cultures

Two-stage batch cultures were performed in 500-mL baffled Erlenmeyer flasks filled with 200 mL of medium. In two-stage batch cultures, the accumulation of intracellular lipids by *Y. lipolytica* W29 cells from VFAs was preceded by a growth phase without VFAs. This first stage occurred in glucose-based medium composed by: 20 g·L^{-1} glucose, 0.5 g·L^{-1} yeast extract, 1.7 g·L^{-1} YNB and 0.503 g·L^{-1} ammonium sulfate for biomass production. After 24 hours of cell growth, corresponding to the final of exponential growth phase, a mixture of acetate, propionate and butyrate, with equal concentration of each one, was added to the medium (second stage). In this strategy, several total VFAs concentration (6 g·L^{-1}, 18 g·L^{-1}, 24 g·L^{-1} and 30 g·L^{-1} of VFAs) were tested. The two-stage batch culture using a mixture with 18 g·L^{-1} of VFAs was also performed with *Y. lipolytica* NCYC 2904.

3.2.4. Bioreactor batch experiments

To evaluate the effect of oxygen volumetric mass transfer coefficient (k_La) on cell growth and lipids accumulation by *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904, several batch cultures were carried out in a 5-L stirred tank bioreactor (BIOSTAT® B Plus, Sartorius) at 27 °C, varying the specific air flow rate (0.5 vvm – 1.5 vvm) and agitation rate (200 rpm – 600 rpm). Yeast cells, pre-grown overnight in YPD medium, were centrifuged and resuspended (at an initial cell density of 0.5 g·L^{-1}) in 2.5 L of VFA-based medium consisted of: VFAs mixture (2 g·L^{-1} of each VFAs), 20 g·L^{-1} glucose, 0.5 g·L^{-1} yeast extract, 1.7 g·L^{-1} YNB and 0.685 g·L^{-1} ammonium sulfate. pH of culture medium was maintained at 6 by the addition of hydrochloric acid 2 M or sodium hydroxide 2 M. Dissolved oxygen concentration was measured with an optical probe (InPro 6860i, Mettler Toledo, USA) and the respective meter (Solaris Biotechnology SRL, Italy).

3.2.5. k_La measurement

To calculate the experimental k_La at different aeration conditions used in yeast cultures, a static gassing-out technique was employed in blank assays (culture medium without cells). This method consists of following the dissolved oxygen concentration in liquid until saturation at specific conditions of air flow and

agitation rates, after a preliminary gassing-out with nitrogen to remove the oxygen in medium (Ferreira et al. 2016).

3.2.6. Analytical methods

At appropriate intervals, culture samples were taken for the measurement of pH, and the quantification of cell mass, glucose, glycerol and VFAs concentration. At the end of experiments, cellular lipids content and long-chain fatty acids(LCFA) were determined and monitored by microscopic visualization.

Cell mass concentration was quantified by optical density at 600 nm and converted to cell dry weight ($g \cdot L^{-1}$) by a calibration curve. Glucose, glycerol and VFAs (acetate, propionate and butyrate) concentration were measured by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (300mm x 7.8mm, 8 µm particle size) at 60 °C and coupled with refractive index and ultra-violet detectors. The mobile phase was sulfuric acid 5 mM at 0.5 $mL \cdot min^{-1}$ flow rate.

Microbial lipids were extracted from lyophilized cells using a mixture of methanol and chloroform and quantified by phospho-vanillin colorimetric method (Lopes et al., 2018). Briefly, ten milligrams of lyophilized cells were suspended in 2 mL of methanol and chloroform (1:1, v/v), vortexed for 3 min and 250 µL of supernatant was collected and heated at 100 °C until the total evaporation of solvents. Then, 100 µL of sulfuric acid 98 % was added and the solution was incubated for 15 min at 100 °C. After cooling to room temperature, 2.4 mL of phospho-vanillin reagent (vanillin dissolved in orthophosphoric acid 85 %) was added and the mixture rested for 15 min. The phospho-vanillin solution reacted with lipids producing a pink color, whose intensity was quantified by measuring the absorbance at 490 nm. This absorbance was converted to lipids concentration ($g \cdot L^{-1}$) by a calibration curve, using olive oil as standard.

The fatty acid composition of microbial lipids was analyzed by gas chromatography (GC). Prior to GC analysis, fatty acids were extracted from lyophilized cells with chloroform and transformed into their methyl esters (FAME) with a mixture of methanol acidified with sulfuric acid (85:15, v/v), using pentadecanoic acid (C15:0) as internal standard (Lopes et al., 2019). The organic phase containing FAMEs was analyzed in a chromatograph (CP-3800 gas chromatograph (Varian Inc., USA)) fitted with FID detector and TRACSIL TR-WAX capillary column (30 m x 0.25 mm x 0.25mm, Teknokroma, Spain). The temperatures of injector and detector were, respectively, 220 °C and 250 °C, and helium at 1 $mL \cdot min^{-1}$ was used as carrier gas. For the complete separation of FAME, a temperature gradient was used: 50 °C for 2 min, followed by an increase of 10 $°C \cdot min^{-1}$ up to 225 °C, which was maintained by 10 min. The identification of FAME was carried out by comparison of retention times with a mixture of FAME standards.

The relative amount of each fatty acid (%) was defined as the ratio between its concentration ($g \cdot L^{-1}$) and the total fatty acids detected in the sample.

Lipids accumulated intracellularly in lipid bodies were visualized by fluorescence microscopy after cells staining with Nile red. A cellular suspension – culture samples were centrifuged, washed twice with PBS 0.1 M and resuspended in PBS 50 mM to a final absorbance of 5 (λ = 600 nm) – was stained with a Nile red solution. The staining solution contained 0.1 $g \cdot L^{-1}$ Nile red in acetone and was added to the cellular suspension in the proportion of 1:10 (v/v) and incubated at room temperature for 1 h. Stained cells were then visualized in an Olympus BX 51 microscope using an exciting wavelength of 470 nm – 490 nm.

3.2.7. Kinetic parameters

The biomass yield ($Y_{X/S}$, $g \cdot g^{-1}$) was calculated according to Equation 3.1, where ΔX is the difference between the biomass concentration at the end of growth (X_f) and initial biomass concentration (X_i), and ΔS is the difference between the initial substrate concentration (S_i) and the substrate concentration at the end of growth (S_f).

$$Y_{X/S} = \frac{\Delta X}{\Delta S} = \frac{X_f - X_i}{S_i - S_f} \quad \text{(Equation 3.1)}$$

The lipids yield ($Y_{L/S}$, $g \cdot g^{-1}$) was calculated according to Equation 3.2, where ΔL is the difference between the lipids concentration at time t (h) (L_f) and the initial lipids concentration (L_i), and ΔS is the difference between the initial substrate concentration (S_i) and the substrate concentration at time (h) (S_f).

$$Y_{L/S} = \frac{\Delta L}{\Delta S} = \frac{L_f - L_i}{S_i - S_f} \quad \text{(Equation 3.2)}$$

The VFAs uptake rate (R_{VFAs}, $g \cdot L^{-1} \cdot h^{-1}$) corresponds to the slope of the linear regression adjusted to VFAs concentration through the time.

3.2.8. Statistical analysis

The results of all experiments are presented as the mean of two independent biological replicates. One-way analysis of variance (ANOVA) was used for the statistical analysis of the data and the significant differences among means ($p < 0.05$) were identified by Tukey's multi-range test (Statgraphics Centurion XVI software, Version 16.2.04).

3.3. Results and Discussion

3.3.1. Batch cultures of *Y. lipolytica* on VFAs-based medium

The ability of *Y. lipolytica* W29 to use VFAs as sole carbon and energy sources to grow and accumulate lipids was firstly evaluated in Erlenmeyer flask experiments. Acetate, propionate and butyrate, which are usually the most common VFAs obtained in acidogenesis step of organic wastes anaerobic digestion (Gameiro et al. 2016), were used in batch cultures. *Yarrowia lipolytica* W29 was able to grow in each VFA, although higher final biomass was attained in butyrate-based medium and no significant differences were observed in yeast growth with acetate and propionate (Figure 3.1A). In fact, the biomass yield (cell mass per VFA mass consumed) obtained with butyrate was 1.8- and 1.6-fold higher than the values attained with acetate and propionate, respectively (Table 3.1). Apparently, increasing VFAs chain length led to the rise of biomass production. Llamas et al. (2020c) also observed that the final cellular concentration of *Y. lipolytica* CECT1240, growing in several VFAs (C2 to C6), increased proportionally with chain length. The maximum biomass obtained in caproate (C6), for example, was approximately 2-fold of that attained in acetate (C2). Moreover, a 3-fold improvement in biomass yield was observed when the authors used butyrate as carbon source, instead of acetate. Studies with other species also concluded that the use of butyrate as carbon source for *Cryptoccocus curvatus* gives rise to higher biomass yield comparatively to acetate and propionate (Zheng et al. 2012; Liu et al. 2017a; Park et al. 2017; Llamas et al. 2020c). By contrast, some works showed that higher cellular growth was obtained in acetate comparatively to other VFAs (Kolouchová et al. 2015; Gao et al. 2017; Gao et al. 2020).

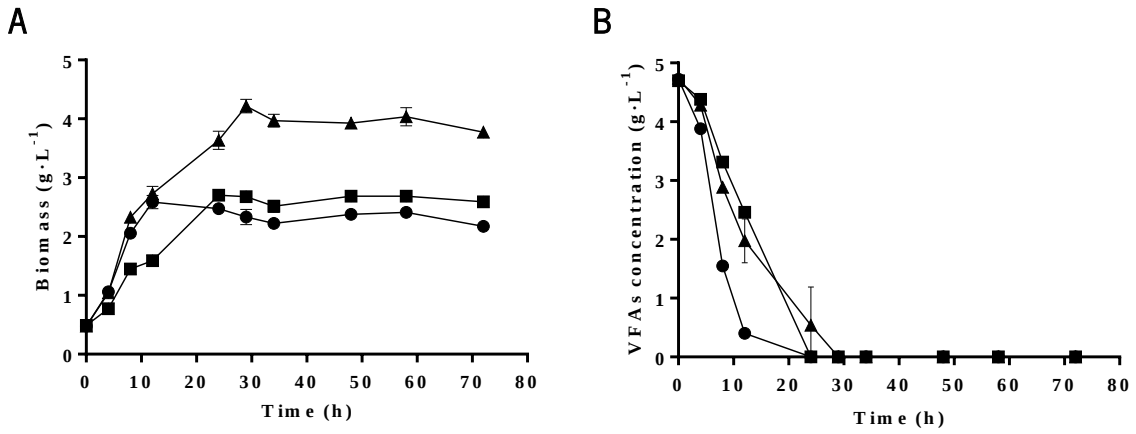

Figure 3.1 - Time course of *Y. lipolytica* W29 growth (A) and VFAs uptake (B) in batch cultures using single VFAs: acetate (●); propionate (■) and butyrate (▲). The error bars represent the standard deviation of two independent replicates.

In all experiments, no lag phase of cellular growth was observed, and the stationary phase was reached approximately after 27 hours of yeast cultivation, which corresponds to the time of complete depletion of each VFAs from culture medium (Figure 3.1B). The consumption profiles show that VFAs consumption is always associated with yeast growth and suggest that acetate was consumed faster than other VFAs. The consumption rates calculated for each VFAs confirm that there were statistical differences between uptake rates of VFAs, and acetate was consumed 47 % faster than other VFAs (Table 3.1). It is worth to notice that uptake rates reported herein are considerable higher than others found in the literature. In *Y. lipolytica* CICC 31596 cultures, 2.5 $g \cdot L^{-1}$ of acetate, propionate and butyrate were totally consumed only after 48 h, 84 h and 96 h, respectively (Gao et al., 2017). Differences in utilization rates of VFAs may be attributed to their different metabolic routes inside *Y. lipolytica* cells. While acetate is converted directly into acetyl-CoA by acetyl-CoA synthase (ACS), propionate is firstly converted to propionyl-CoA that enters in the methyl citrate cycle to produce pyruvate, which is then decarboxylated to acetyl-CoA. Butyrate is converted to butyryl-CoA and then to acetyl-CoA via β-oxidation. The acetyl-CoA produced from all VFAs can be directly incorporated into lipids synthesis or transported into the mitochondria and enter in the TCA cycle for energy production. Acetyl-CoA units can also incorporate the glyoxylate shunt and gluconeogenesis pathway (Liu et al., 2016; Vajpeyi and Chandran, 2015).

The highest lipid production was reached in butyrate-based medium (Table 3.1), that was also reported for other yeast species, in which butyrate in relatively low concentrations was more suitable for lipids synthesis by *C. curvatus* than acetate and propionate (Liu et al. 2017a; Park et al. 2017). In high alkaline conditions (pH 10), butyrate was also the best substrate for lipids production by *Y. lipolytica* CICC 31596 (Gao et al. 2020). By contrast, in slightly acidic conditions (pH 6), a 13 % and 38 % enhancement on lipids synthesis was attained in acetate comparatively with propionate and butyrate, respectively (Gao et al., 2017).

Table 3.1 - Values of biomass yield ($Y_{X/S}$), lipids yield ($Y_{L/S}$), VFAs uptake rate (R_{VFAs}), lipids content and lipids concentration obtained in *Y. lipolytica* W29 batch cultures carried out with each VFA, with addition of glucose (5 g·L^{-1}) to each VFA-based medium and with a mixture of 6 g·L^{-1} total VFAs with glucose (20 g·L^{-1}) or glycerol (20 g·L^{-1}). Data are average ± standard deviation for two independent replicates. Values followed by the same letter do not present statistically significant differences ($p \geq 0.05$).

Carbon source	$Y_{X/S}$ (g·g^{-1})	$Y_{L/S}$ (g·g^{-1})	R_{VFAs} (g·L^{-1}·h^{-1})	Lipids content (%, w/w)	Lipids concentration (g·L^{-1})
Acetate	0.40 ± 0.01 ^{a}	0.037 ± 0.001 ^{ab}	0.38 ± 0.02 ^{a}	7.3 ± 0.4 ^{a}	0.17 ± 0.01 ^{a}
Propionate	0.46 ± 0.01 ^{ab}	0.045 ± 0.002 ^{ab}	0.20 ± 0.00 ^{b}	7.9 ± 0.6 ^{ab}	0.21 ± 0.01 ^{a}
Butyrate	0.729 ± 0.002 ^{c}	0.096 ± 0.005 ^{c}	0.164 ± 0.001 ^{c}	11.5 ± 0.5 ^{cd}	0.45 ± 0.02 ^{b}
Acetate + Glucose	0.54 ± 0.01 ^{bd}	0.050 ± 0.003 ^{bd}	0.34 ± 0.00 ^{a}	9.1 ± 1.1 ^{be}	0.46 ± 0.03 ^{b}
Propionate + Glucose	0.39 ± 0. 03 ^{a}	0.045 ± 0.008 ^{ab}	0.132 ± 0.003 ^{d}	9.6 ± 1.3 ^{e}	0.41 ± 0.07 ^{b}
Butyrate + Glucose	0.72 ± 0.01 ^{c}	0.090 ± 0.001 ^{c}	0.202 ± 0.005 ^{b}	12.4 ± 1.2 ^{d}	0.85 ± 0.01 ^{c}
VFAs + Glucose	0.63 ± 0.07 ^{cd}	0.06 ± 0.02 ^{d}	0.21 ± 0.01 ^{b}	10 ± 2 ^{bce}	1.42 ± 0.31 ^{d}
VFAs + Glycerol	0.41 ± 0.03 ^{a}	0.033 ± 0.002 ^{a}	0.16 ± 0.01 ^{c}	7.9 ± 0.4 ^{abe}	0.82 ± 0.02 ^{c}

In general, oleaginous yeasts have the ability to accumulate more than 20 % of their cell dry weight as intracellular lipids. The content, concentration and yield of lipids on Table 3.1 were low comparatively to others reported in the literature for microbial lipids production by *Y. lipolytica* strains from VFAs (Fontanille et al. 2012; Gao et al. 2017), glucose (Katre et al. 2012), animal fat (Lopes et al., 2018), waste cooking oils (Lopes et al., 2019) or glycerol (Poli et al. 2014; Sara et al. 2016). In these substrates, lipids content ranges from 20 % (w/w) to 60 % (w/w), lipids concentration varies from 0.6 g·L^{-1} to 14 g·L^{-1} and lipids yield ranges from 0.1 g·g^{-1} to 0.2 g·g^{-1}. Though VFAs have higher theoretical conversion efficiencies and shorter metabolic pathways for lipid synthesis compared with other substrates (Zheng et al. 2012; Gong et al. 2016), many nutritional and environmental factors, such as VFAs concentration, pH and yeast strain, can strongly affect lipids accumulation (Katre et al. 2012; Gao et al. 2017; Gao et al. 2020).

The pH of culture medium increased during lipids production over time to values of 9, due to VFAs consumption. At slightly acidic pH, VFA is mostly in undissociated form, which once inside the cell,

dissociates intracellularly, and leads to cytosolic acidification, which may alter several metabolic pathways (Casal et al. 2008). In this turn, the adverse effects of VFAs at low concentration are weak and negligible (Fei et al. 2011b; Gao et al. 2017; Liu et al. 2017a).

Despite of *Y. lipolytica* W29 readily consume VFAs, the addition of a co-substrate (5 g·L^{-1} glucose) to each VFAs-based medium was tested as an attempt to improve yeast growth and, consequently, the titer of lipids produced. Approximately 2- fold improvement on cellular concentration was observed with the addition of glucose, regardless of the VFAs in the medium (Figure 3.2A). Although no differences were observed on yield biomass (cell mass per mass of total substrates, VFAs and co-substrate, consumed) with the addition of glucose, the highest biomass production was attained with butyrate as occurred in experiments with single VFAs (Table 3.1).

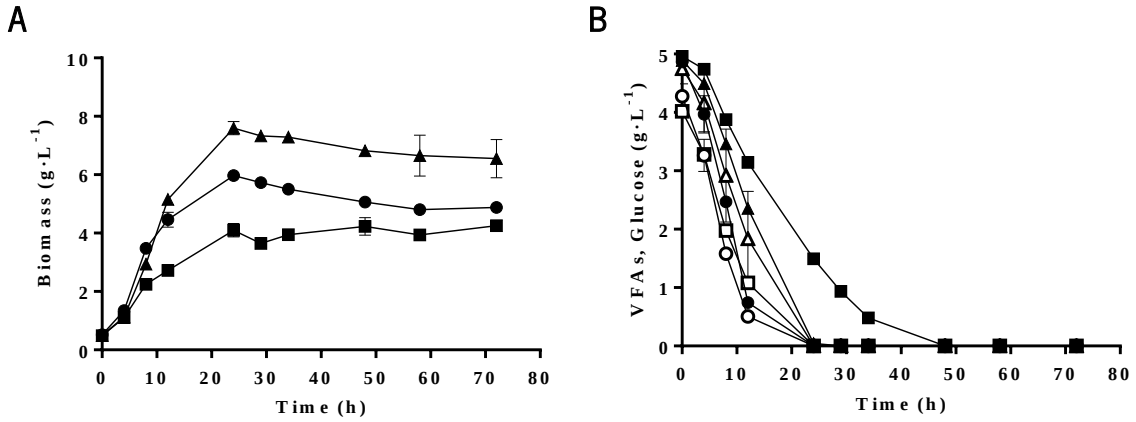

Figure 3.2 -Time course of cell growth (A) and VFAs (close symbols) and glucose uptake (open symbols) (B) obtained in *Y. lipolytica* W29 batch cultures in VFA (5 g·L^{-1})-based medium supplemented with glucose (5 g·L^{-1}): acetate (●,○); propionate (■,□) and butyrate (▲,Δ). The error bars represent the standard deviation of two independent replicates.

In all experiments, cell proliferation started without lag phase and the stationary phase was attained after 24 hours of yeast cultivation, similarly to the experiments with single VFAs (27 hours). The addition of a co-substrate, easily assimilable by *Y. lipolytica,* changed the growth profile of the yeast. The specific growth rate on acetate (0.185 h^{-1} ± 0.002 h^{-1}), propionate (0.147 h^{-1} ± 0.003 h^{-1}) and butyrate (0.200 h^{-1} ± 0.001 h^{-1}) were 22 %, 33 % and 25 %, respectively, higher than those obtained in the absence of glucose. In the presence of glucose, all substrates (VFAs + glucose) were consumed simultaneously with yeast growth, but the consumption of propionate was slower than other VFAs and glucose, and only after 48 h (stationary growth phase) was completely depleted from the culture medium (Figure 3.2B). By contrast,

acetate, butyrate and glucose were totally consumed after 24 h of cultivation, showing that *Y. lipolytica* W29 can effectively utilize VFAs and sugars simultaneously for cellular growth. In butyrate-based medium, the addition of glucose led to an increase of VFAs uptake rate, whereas in other VFAs media a decrease was observed with glucose supplementation (Table 3.1). Furthermore, it is worth to notice that uptake rates of acetate and glucose were similar and higher than propionate and butyrate uptake rates (Table 3.1).

An improvement on lipids cellular content was observed by the addition of glucose as co-substrate, in cultures with acetate and propionate but was not affected for butyrate-based media. Around 1.2-fold enhancement on lipids production was obtained with the supplementation of glucose in acetate and propionate media. Glucose, under nitrogen limited condition, is metabolized by *Y. lipolytica* through glycolysis and tricarboxylic acid cycle to generate acetyl-CoA, which is then used for *de novo* fatty acid synthesis (Papanikolaou et al. 2009). Although the lipids content remained low (12.4 %), the increase of final biomass with the addition of glucose led to a 2- and 3-fold enhancement of microbial lipids concentration (0.41 $g \cdot L^{-1}$ – 0.85 $g \cdot L^{-1}$) and consequently a 10-fold improvement in lipids productivity comparatively with experiments with butyrate, propionate and acetate individually (Table 3.1). In previous works, the addition of glucose as co-substrate to acetate and propionate led to an enhancement of lipids content from 13.3 % (w/w) to 16.5 % (w/w) and from 8.9 % (w/w) to 10.2 % (w/w), respectively (Kolouchová et al. 2015). The lipids production by *Y. lipolytica* from frying vegetable oils enhanced from 25 % (w/w) to 55 % (w/w) when medium was supplemented with glucose (Bialy et al. 2011).

Since cellular growth was enhanced by addition of glucose and, consequently, the amount of lipids synthesized also increased (Table 3.1), the raise of glucose concentration up to 20 $g \cdot L^{-1}$ was tested as an attempt to boost even more the concentration of biomass and microbial lipids. Additionally, the replacement of glucose by glycerol, a low-cost by-product of biodiesel industry, was tested. As the liquid broth of a real anaerobic digestion of organic wastes consists in a mixture of VFAs (Vajpeyi and Chandran 2015; Gao et al. 2017; Strazzera et al. 2018), batch cultures with a mixture of VFAs (with 2 $g \cdot L^{-1}$ acetate, 2 $g \cdot L^{-1}$ propionate and 2 $g \cdot L^{-1}$ butyrate) and 20 $g \cdot L^{-1}$ glucose or 20 $g \cdot L^{-1}$ glycerol were carried out. The mixture of VFAs was chosen as model based on total initial concentration of 6 $g \cdot L^{-1}$ of VFAs. Besides *Y. lipolytica* W29, also *Y. lipolytica* NCYC 2904 was studied in these experiments. This strain, isolated from a maize-processing plant (Illinois, USA), is less explored than W29 strain, though have already been demonstrated its potential to accumulate lipids from food processing by-products like okara (Vong et al. 2016).

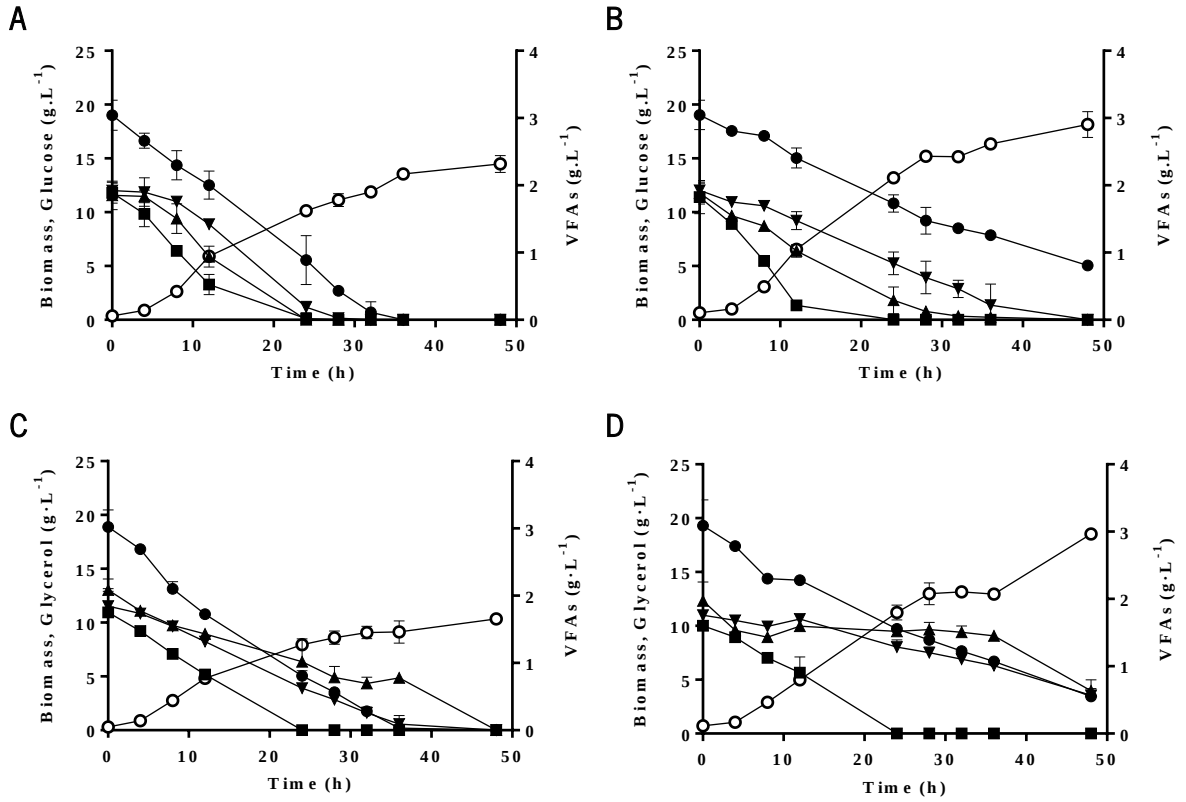

Figure 3.3 - Time course of cell growth (o) and substrate uptake by *Y. lipolytica* W29 (left column) and *Y. lipolytica* NCYC 2904 (right column) in batch cultures with a mixture of 6 g·L^{-1} total VFAs (2 g·L^{-1} of each one: acetate (■), propionate (▲) and butyrate (▼)) with 20 g·L^{-1} glucose (●, A and B) or 20 g·L^{-1} glycerol (●, C and D). The error bars represent the standard deviation of two independent replicates.

In general, the simultaneous increase of glucose concentration and the use of a VFAs mixture did not affects the biomass yield. However, higher biomass concentration (14.5 g·L^{-1} ± 0.6 g·L^{-1}) was attained comparatively to previous experiments (Figure 3.3A). The supplementation of 20 g·L^{-1} glucose essentially prevented pH changes when the yeast is growing on VFAs and thus improved the cellular growth. For *Y. lipolytica* growing on VFAs, the pH of culture medium increases to values above of 9, while in sugar media, the culture pH usually drops due to the production of organic acids (Papanikolaouet al., 2009). *Yarrowia lipolytica* NCYC 2904 was also able to grow in a mixture of VFAs supplemented with glucose with higher yield biomass (0.93 g·g^{-1} ± 0.05 g·g^{-1}), attaining high cell density comparatively to W29 strain cultures (Figure 3.3A and 3.3B). In experiments with glycerol as co-substrate (Figure 3.3C and 3.3D), the same behavior was observed and higher cellular concentration was reached in *Y. lipolytica* NCYC 2904 cultures. Moreover, the biomass yield was comparable to that obtained with glucose, whereas for W29 strain a significantly decrease was observed (Table 3.1). Glucose and VFAs were consumed

simultaneously with yeasts growth, though W29 and NCYC 2904 strains had different consumption profiles. Whereas a complete depletion of glucose and VFAs were observed, respectively, after 36 h and 28 h for *Y. lipolytica* W29 (Figure 3.3A), NCYC 2904 strain did not consume all glucose in the experiments time and the exhaustion of VFAs was observed only after 36 h (Figure 3.3B). The global uptake rate of VFAs by *Y. lipolytica* NCYC 2904 was 24 % lower than that attained by W29 strain. Similarly, glycerol was not totally consumed by NCYC 2904 strain (Figure 3.3D), whereas in W29 strain cultures, this co-substrate was depleted from medium after 40 h (Figure 3.3C). Moreover, the global uptake rate of VFAs were significantly lower in cultures with addition of glycerol than with glucose. Both yeasts showed a remarkable preference for acetate over propionate and butyrate, particularly the NCYC 2904 strain in cultures with glycerol. Furthermore, it is worth to notice that acetate was always consumed faster than other VFAs, regardless of experiments have been carried out with single VFAs or mixed with addition of glucose or glycerol. This is not surprising, since VFAs consumption is related with different metabolic pathways inside yeast cells, and this is not affected by the glucose or glycerol supplementation.

Although the low content of microbial lipids accumulated by *Y. lipolytica* W29 and NCYC 2904 strains of 10 % (w/w) and 13 % (w/w) respectively, an enhancement of lipids concentration as well as lipid productivity (30 $mg \cdot L^{-1} \cdot h^{-1} \pm 6\ mg \cdot L^{-1} \cdot h^{-1}$) were attained with the addition of glucose. For *Y. lipolytica* W29, a 3- and 2-fold improvement on intracellular lipids concentration was obtained, relatively to the experiments with butyrate as sole carbon source and butyrate supplemented with glucose 5 $g \cdot L^{-1}$, respectively (Table 3.1). The amount of lipids produced by *Y. lipolytica* NCYC 2904 ($2.6\ g \cdot L^{-1} \pm 0.4\ g \cdot L^{-1}$) was 45 % higher than that obtained with W29 strain. Additionally, the lipids yield was 2-fold of that obtained with W29 strain, with an average lipid productivity of $54\ mg \cdot L^{-1} \cdot h^{-1} \pm 8\ mg \cdot L^{-1} \cdot h^{-1}$. The lipids yield ($0.144\ g \cdot g^{-1} \pm 0.005\ g \cdot g^{-1}$) and lipids concentration ($2.69\ g \cdot L^{-1} \pm 0.04\ g \cdot L^{-1}$) obtained for *Y. lipolytica* NCYC 2904, using glycerol as co-substrate, were 4.4- and 3.3-fold higher than those reached by W29 strain (Table 3.1), indicating the potential of this yeast NCYC 2904 strain to synthetize lipids from VFAs. Though no significant differences on lipids production were observed between glucose and glycerol cultures of *Y. lipolytica* NCYC 2904, higher lipids productivity was obtained in glucose for W29 strain (Table 3.1).

Fatty acids composition of lipids produced from hydrophilic substrates is dependent on yeast specificity, substrates, as well as culture conditions. In this work, lipids produced by *Y. lipolytica* from VFAs are mainly composed by palmitic, palmitoleic, stearic, oleic and linoleic acids (Table 3.2). Regardless of yeast strain, VFAs type and concentration and presence of glucose or glycerol, lipids produced have a significant higher unsaturated fraction (> 73 %, $p < 0.05$) than saturated one, being composed of oleic (34 % - 45 %) and linoleic acids (20 % - 50 %). However, the relative percentage of each fatty acid was dependent on

substrates used. While using VFAs as sole carbon source *Y. lipolytica* W29 accumulated more oleic and linoleic acids, the addition of glucose or glycerol led to an enhancement of palmitic acid, a slight increase of oleic acid and a decrease of linoleic acid contents. Papanikolaou et al. (2009) reported that the increase of glucose concentration induced an increase of linoleic acid, but a decrease on linoleic acid (20 %) was observed in batch cultures with mixture of VFAs (6 $g \cdot L^{-1}$) and glucose (20 $g \cdot L^{-1}$). It is worth to notice that margaric acid is only synthetized when propionate is used as carbon source (individually or in a mixture with other VFAs). Since the metabolic pathways of lipids synthesis from all VFAs involve acetyl-CoA, fatty acids composition of microbial lipids from different VFAs are expected to be similar. However, propionate is metabolized via a different pathway, and this precursor (propionyl-CoA) synthetizes odd number fatty acids, such as margaric acid (Kolouchová et al. 2015; Gao et al. 2017). This result is utmost important, since odd chain fatty acids may improve the properties of biodiesel and be used as precursors for manufacturing agricultural chemicals (biocides), flavors and industrial chemicals (Park et al. 2018; Bhatia et al. 2019). Although the fatty acids profile of yeast strains tested herein indicated to depend on culture conditions, similar fatty acids compositions were reported to other *Y. lipolytica* strains using VFAs as carbon source (Gao et al. 2017; Gao et al. 2020). In other hydrophilic substrates, such as glycerol (Poli et al. 2014) and hydrophobic carbon sources, such as pork lard (Lopes et al. 2018) and waste cooking oils (Lopes et al. 2019), *Y. lipolytica* strains also accumulated preferentially oleic and linoleic acids, suggesting that lipogenic features of this yeast prevail over substrate nature. Other oleaginous yeasts, such as *Cryptococcus albidus* (Vajpeyi and Chandran 2015) and *Rhodosporidium toruloides* (Huang et al. 2016), growing in VFAs-based media, produced lipids with a similar fatty acids composition of those obtained in this work.

Table 3.2 - Fatty acids composition of microbial lipids produced by *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 in batch cultures. Data are average ± standard deviation for two independent replicates.

Strain	Carbon Source	Relative fatty acid content (%)					
		C16:0	C16:1	C17:0	C18:0	C18:1	C18:2
W29	Acetate	3.8 ± 0.3	10 ± 1	-	1.0 ± 0.8	35.6 ± 0.6	49.6 ± 0.9
	Propionate	2.9 ± 0.6	11.8 ± 0.4	6.1 ± 0.5	4.0 ± 0.9	34.4 ± 0.9	40.7 ± 0.9
	Butyrate	8.3 ± 0.3	10.5 ± 0.2	-	4.8 ± 0.7	41.0 ± 0.6	36.7 ± 0.4
	Acetate + Glucose	7.9 ± 0.2	11.0 ± 0.3	-	2.0 ± 0.5	38.0 ± 0.6	41 ± 1
	Propionate + Glucose	13 ± 2	10.2 ± 0.2	4.9 ± 0	4.0 ± 0.5	35.4 ± 0.4	32 ± 1
	Butyrate + Glucose	10.5 ± 0.1	9.9 ± 0.3	-	3.8 ± 0.5	43.0 ± 0.9	33 ± 1
	VFAs + Glucose	17 ± 1	8.9 ± 0.3	2.9 ± 0.4	8 ± 3	44 ± 4	20 ± 5
	VFAs + Glycerol	12.6 ± 0.8	4.8 ± 0.2	5.1 ± 0.4	6.5 ± 0.3	36 ± 1	35 ± 1
NCYC 2904	VFAs + Glucose	13.2 ± 0.3	9.9 ± 0.3	2.4 ± 0.1	4.7 ± 0.3	43 ± 1	27 ± 2
	VFAs + Glycerol	11.9 ± 0.5	7.1 ± 0.3	2.4 ± 0.2	5.8 ± 0.4	45 ± 1	28 ± 2

3.3.2. Two-stage batch cultures

As was concluded above, the enhancement of cellular growth, by addition of glucose, lead to an increase of lipids production. Thus, a two-stage batch culture was performed with a 24 h first stage of yeast growth on glucose before the addition of VFAs into the culture medium. Mixtures of acetate, propionate and butyrate with 2 $g \cdot L^{-1}$, 6 $g \cdot L^{-1}$, 8 $g \cdot L^{-1}$ and 10 $g \cdot L^{-1}$ of each one, were added to the medium in the second stage. In general, VFAs derived from organic wastes ranged from 4 $g \cdot L^{-1}$ to 40 $g \cdot L^{-1}$ (Gameiro et al. 2016; Gao et al. 2017; Moretto et al. 2019b; Gao et al. 2020; Llamas et al. 2020c).

In general, *Y. lipolytica* W29 growth and biomass yield diminished with the increase of VFAs concentration, with approximately a 2-fold decrease by raising the total amount of VFAs from 6 $g \cdot L^{-1}$ to 30 $g \cdot L^{-1}$ (Figure 3.4A; Table 3.3). The adverse effects of VFAs on yeast cells are commonly caused by undissociated form of VFAs, which leads to intracellular acidification and induces stress on cell metabolism. Although yeasts possess a series of regulatory mechanisms to maintain a near neutral intracellular environment, high expenditure of ATP could interfere with membrane transport of phosphate and consequently affect yeast

growth (Rodrigues and Pais 2000; Gao et al. 2017; Gao et al. 2020). According to Fei et al. (2011b) and Fontanille et al. (2012), this inhibition was observed for an initial concentration of VFAs above 5 g·L^{-1}. Moreover, different VFA types and initial concentrations could exert different intensities of inhibition. For instance, Gao et al. (2017) reported that high concentrations of VFAs inhibited cell growth in the following order: butyric acid > propionic acid > acetic acid. While *Y. lipolytica* CICC 31596 was able to grow on batch cultures with 20 g·L^{-1} acetic acid, no cellular growth was observed with 20 g·L^{-1} propionic acid and with concentrations above 10 g·L^{-1} of butyric acid. VFAs ratio can also affects cellular growth, since the predominance of short-chain VFAs (e.g., acetic acid) in the culture medium has a positive effect on yeast growth, on the contrary to the presence of high proportion of long-chain VFAs (propionic and butyric acid) (Fei et al., 2011b; Llamas et al., 2020). Despite higher VFAs concentrations had affected adversely yeast growth, *Y. lipolytica* W29 was able to consume completely all VFAs, even in the experiments performed with 24 g·L^{-1} and 30 g·L^{-1} (Figure 3.4B). Faster consumption and VFAs uptake rate were obtained with 6 g·L^{-1}, but no significant differences were observed for results with VFAs concentration between 18 g·L^{-1} and 30 g·L^{-1} (Table 3.3). It is worth to notice that VFAs uptake rates attained in this work were considerable higher than others reported in literature. In *Y. lipolytica* CECT1240 cultures, 10.5 g·L^{-1} of VFAs mixture was totally consumed after 250 h, while for 26.5 g·L^{-1} VFAs mixture, only 7.6 g·L^{-1} was consumed after 380 h of culture (Llamas et al. 2020c).

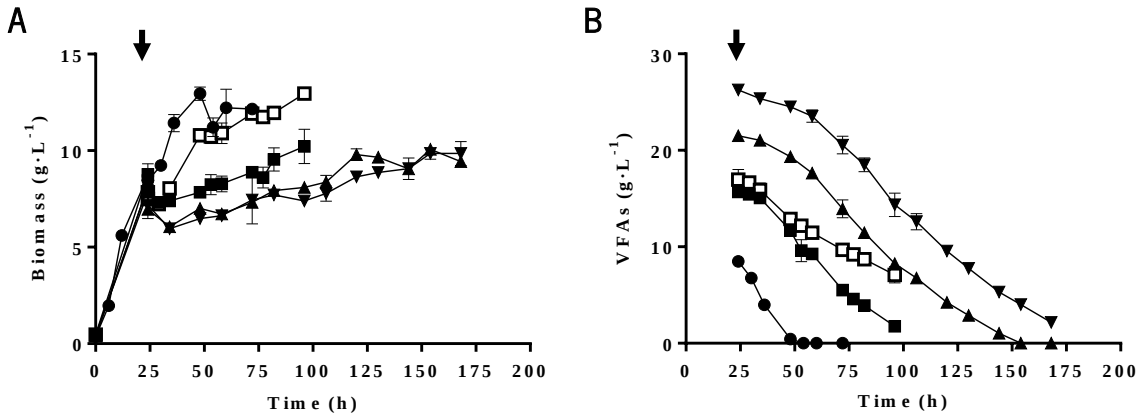

Figure 3.4 - Time course of cellular growth (A) and total VFAs uptake (B) in two-stage batch cultures of *Y. lipolytica* W29 (close symbols) and *Y. lipolytica* NCYC 2904 (open symbols). After 24 hours of cellular growth in glucose (20 g·L^{-1}), mixtures of 6 g·L^{-1} (●), 18 g·L^{-1} (■, □), 24 g·L^{-1} (▲) and 30 g·L-1 (▼) of total VFAs were added to culture medium. The error bars represent the standard deviation of two independent replicates.

No improvement in biomass and lipids production was attained with the two-stage batch cultures of *Y. lipolytica* W29 using mixture with a total VFAs concentration of 6 g·L^{-1} (Table 3.3) relatively to batch culture of mixture of VFAs (6 g·L^{-1}) with glucose (Table 3.1). However, the global VFAs uptake rate of VFAs on two-stage batch culture (0.28 g·L^{-1}·h^{-1}) was 25 % higher ($p < 0.05$) than that obtained in batch culture (0.21 g·L^{-1}·h^{-1}), due to the increased cellular concentration of the culture.

Table 3.3 - Values of biomass yield ($Y_{X/S}$), lipids yield ($Y_{L/S}$), total VFAs uptake rate (R_{VFAs}), lipids content and lipids concentration obtained in two-stage batch cultures of *Y. lipolytica* W29. After 24 h of cellular growth in glucose (20 g·L^{-1}), mixtures of 6 g·L^{-1}, 18 g·L^{-1}, 24 g·L^{-1} and 30 g·L^{-1} of VFAs were added to culture medium. Data are average ± standard deviation of two independent replicates. Values followed by the same letter do not present statistically significant differences ($p \geq 0.05$).

VFAs Concentration	$Y_{X/S}$ (g·g^{-1})	$Y_{L/S}$ (g·g^{-1})	R_{VFAs}* (g·L^{-1}·h^{-1})	Lipids content (%, w/w)	Lipids concentration (g·L^{-1})
6 g·L^{-1}	0.38 ± 0.02 ^{a}	0.058 ± 0.006 ^{a}	0.28 ± 0.01 ^{a}	13.8 ± 1.4 ^{a}	1.78 ± 0.16 ^{a}
18 g·L^{-1}	0.26 ± 0.02 ^{b}	0.068 ± 0.002 ^{a}	0.194 ± 0.002 ^{b}	25.4 ± 0.9 ^{b}	2.3 ± 0.2 ^{b}
24 g·L^{-1}	0.20 ± 0.01 ^{c}	0.042 ± 0.004 ^{b}	0.165 ± 0.001 ^{b}	17.5 ± 1.6 ^{c}	1.28 ± 0.09 ^{c}
30 g·L^{-1}	0.20 ± 0.01 ^{c}	0.045 ± 0.006 ^{b}	0.167 ± 0.000 ^{b}	16.9 ± 1.8 ^{c}	1.26 ± 0.15 ^{c}

*Values correspond to second stage of cultivation.

Though the highest biomass yield was obtained in cultures with lower amount of VFAs, the highest lipids yield, lipids content and lipids concentration were attained at total VFAs concentration of 18 g·L^{-1}. In cultures with 24 g·L^{-1} and 30 g·L^{-1} of total VFAs, great amounts of VFAs in unionized form are present, which may affect the activity of some enzymes involved in lipid synthesis (Gao et al., 2017). Gong et al. (2016) also observed a positive effect of increased acetate concentration up to 20 g·L^{-1} on lipids synthesis by *C. curvatus* ATCC 20509, but above this amount the accumulation of lipids decreased considerably. Results obtained in two-stage batch culture with VFAs mixture of 30 g·L^{-1} were similar to attained in batch culture with 30 g·L^{-1} of acetate (Gao et al. 2020). Moreover, the authors showed that initial pH of culture medium had a crucial effect on yeast when high concentration of acetate was used and increasing pH to alkaline levels can alleviate the toxic effect of high content of VFAs. Thus, the slight acidic pH of culture medium in beginning of second stage (after addition of VFAs) could be contributed to inhibition of high VFAs concentration on lipids production.

As the two-stage batch cultures using a mixture of 18 $g \cdot L^{-1}$ VFAs proved to be effective for lipids synthesis enhancement by *Y. lipolytica* W29, and NCYC 2904 strain produced 41 % higher lipids than W29 strain in batch cultures, this strategy was also tested with NCYC 2904 strain. The biomass yield (0.46 $g \cdot g^{-1}$ ± 0.07 $g \cdot g^{-1}$) was higher than that for W29 strain, though total VFAs uptake rate (0.138 $g \cdot L^{-1} \cdot h^{-1}$ ± 0.002 $g \cdot L^{-1} \cdot h^{-1}$) be considerably lower, and approximately 7 $g \cdot L^{-1}$ of VFAs remained in culture medium at the end of growth. In the beginning of second stage (addition of VFAs), 10 $g \cdot L^{-1}$ of glucose remained in *Y. lipolytica* NCYC 2904 cultivation medium, whereas in W29 cultures only 4 $g \cdot L^{-1}$ of glucose were not consumed. This may be the reason for the lower uptake rate of VFAs mixture obtained for NCYC 2904 strain. At the end of experiments, the lipids content (26.7 % ± 1.6 %, w/w) and lipids concentration (3.5 $g \cdot L^{-1}$ ± 0.2 $g \cdot L^{-1}$), were approximately 2-fold higher than those attained by W29 strain, as occurred in batch cultures.

It is worth to notice that the amount of lipids produced in two-stage batch cultures of NCYC 2904 and W29 strains, with addition of 18 $g \cdot L^{-1}$ of VFAs mixture, led to a 2- and 2.5-fold improvement on lipids content comparatively to that obtained in batch cultures with a mixture of VFAs (6 $g \cdot L^{-1}$) and glucose (20 $g \cdot L^{-1}$). Although few authors have tested similar strategies for lipids production from VFAs by oleaginous yeasts in flask-scale, the results obtained herein are comparable or even higher than others found in the literature for *C. curvatus*, (Christophe et al. 2012), *C. albidus* (Fei et al. 2011a) and *R. toruloides* (Huang et al. 2016)

The microscopic visualization of *Y. lipolytica* W29 (Figure 3.5A) and *Y. lipolytica* NCYC 2904 (Figure 3.5C) cells cultivated in medium with VFAs revealed that both yeast cells exhibited a typical oval form. Additionally, it was possible to clearly visualize the lipids accumulated in lipid bodies within the cells. The fluorescence microscopy after staining with Nile Red allows the clear visualization of lipids accumulated in lipid bodies, stained in yellow. *Yarrowia lipolytica* W29 cells (Figure 3.5B) had one or two lipid bodies within the cells, while in *Y. lipolytica* NCYC 2904 cells (Figure 3.5D) several lipid bodies inside the cells were observed, which is in accordance with higher lipid content reached by NCYC 2904 strain comparatively to W29 strain.

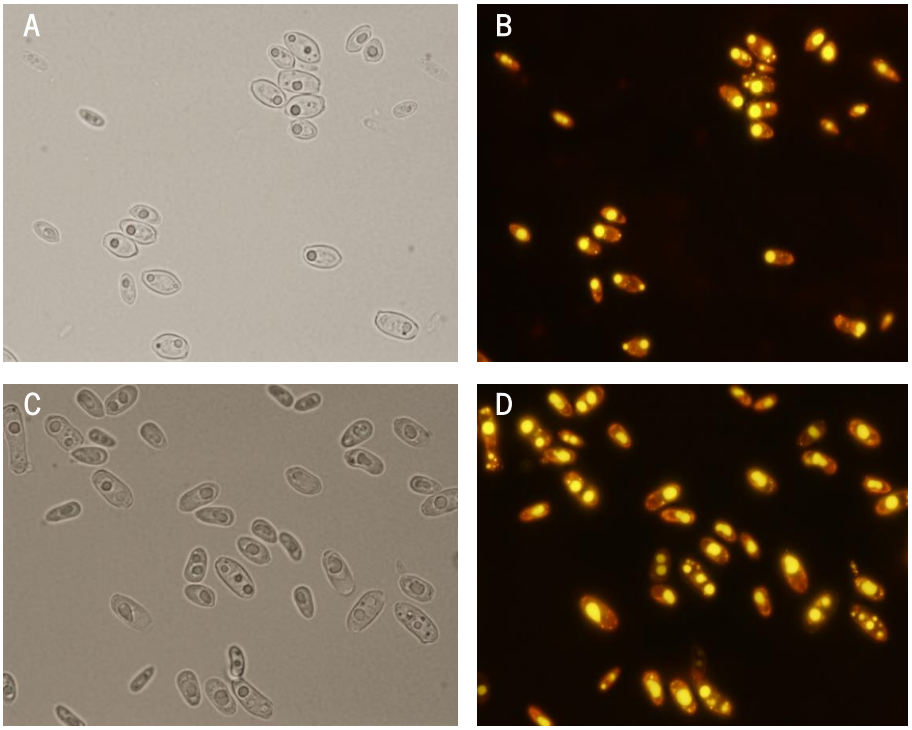

Figure 3.5 - Light microscopy (left column) and fluorescence microscopy images (right column) of *Y. lipolytica* W29 (A, B) and *Y. lipolytica* NCYC 2904 (C, D) cells stained with Nile red: lipid bodies visualization after growth on two-stage batch culture with 18 $g \cdot L^{-1}$ VFAs (Magnification 1000 x).

On whole, Erlenmeyer flask experiments demonstrated the ability of *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 to accumulate lipids using VFAs as carbon source, and both strains showed high tolerance for assimilate VFAs at concentrations up to 18 $g \cdot L^{-1}$ in two-stage batch cultures. In this strategy, lipids produced by both strains are mainly composed by oleic acid (> 50 %), followed by linoleic and stearic acids (Table 3.4). It should be noticed that the unsaturated fraction (72 % - 74 %) largely exceeded the saturated one (17 % - 22 %), making these oils a plausible substitute of vegetable oils for biodiesel production.

Table 3.4 - Fatty acids composition of microbial lipids produced by *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 in two-state batch cultures with addition of 18 g·L^{-1} of mixed VFAs. Data are average ± standard deviation for two independent replicates.

Strain	Relative fatty acid content (%)					
	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2
W29	11.7 ± 0.6	5.3 ± 0.1	3.0 ± 0.1	7.5 ± 0.7	51.0 ± 0.5	15.3 ± 0.1
NCYC 2904	9.6 ± 0.2	6.2 ± 0.0	2.2 ± 0.2	5.6 ± 0.2	53.8 ± 0.2	14.0 ± 0.2

3.3.3. Effect of oxygen supply on microbial lipids production

Dissolved oxygen concentration in cultivation medium is an operational parameter with great influence on *Y. lipolytica* growth and lipids production (Magdouli et al. 2018). Furthermore, oxygen mass transfer from gas phase to the liquid medium is a crucial variable for process scale-up, since *Y. lipolytica* is a strictly aerobic yeast. Therefore, the effect of k_La on growth and lipids synthesis by *Y. lipolytica* from VFAs was studied. Both W29 and NCYC 2904 strains were studied in batch cultures in a STR lab-scale bioreactor using a medium with a mixture of 6 g·L^{-1} total VFAs and glucose (20 g·L^{-1}). Different aeration and agitation rates were varied to obtain three different values of k_La of 22 h^{-1} ± 2 h^{-1} (at 0.5 vvm and 200 rpm), 87 h^{-1} ± 2 h^{-1} (at 1 vvm and 400 rpm) and 125 h^{-1} ± 0 h^{-1} (at 1.5 vvm and 600 rpm). During the first hours of yeast cultivation (corresponding to the exponential growth phase), a decrease on oxygen concentration in the medium was observed, particularly in experiments carried out at k_La of 22 h^{-1} and 87 h^{-1}. In condition of lower oxygen transfer efficiency, oxygen concentration dropped to zero in the first hour of growth, remaining in this value until the end of process. This can justify the low biomass production and VFAs consumption rate obtained in this condition, owing to an oxygen limitation (Figure 3.6A and 3.6B). In the experiments conducted at k_La of 87 h^{-1}, the dissolved oxygen concentration dropped to zero (W29 strain) and to 8 % (NCYC 2904 strain) in the first hours of cultivation, increased during stationary phase of growth and remained above 80 % (W29) and 70 % (NCYC 2904) until the end of experiments. Contrariwise, for k_La of 125 h^{-1}, the dissolved oxygen concentration never fell to zero and stabilized at around 95 % after exponential growth phase.

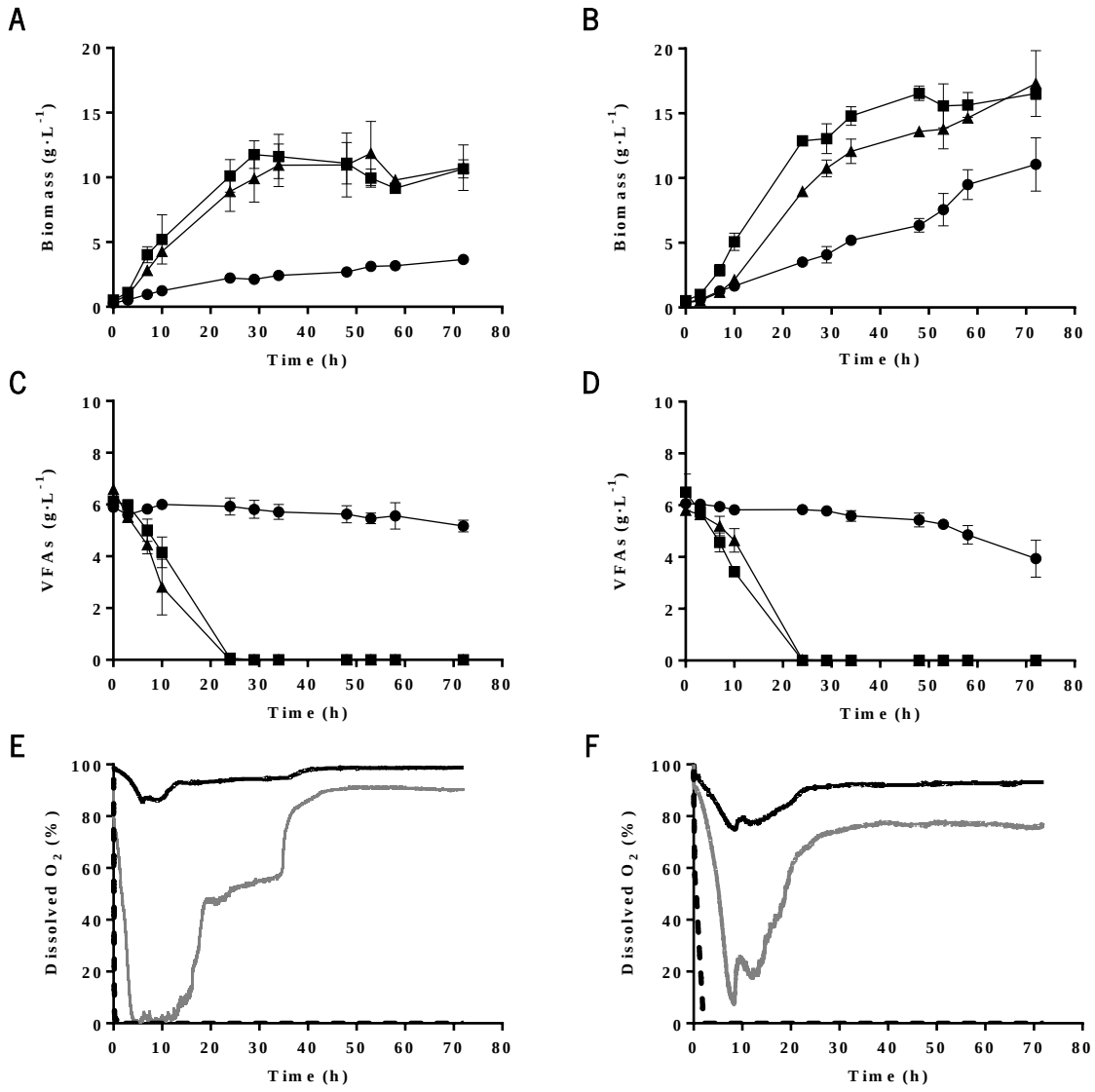

Figure 3.6 - Time course of cell growth (A, B), total VFAs uptake (C, D) and dissolved oxygen concentration (E, F) obtained in batch cultures of *Y. lipolytica* W29 (left column) and *Y. lipolytica* NCYC 2904 (right column) carried out in a STR bioreactor at different k_La conditions: 22 h^{-1} (●, black dashed line), 87 h^{-1} (▲, grey line) and 125 h^{-1} (■, black line). The error bars represent the standard deviation of two independent replicates.

The increase of K_La from 22 h^{-1} to 87 h^{-1} had a positive effect on cellular growth of *Y. lipolytica* W29 and NCYC 2904, but the further increase did not favor cellular growth and VFAs consumption (Table 3.5). As *Y. lipolytica* is a strictly aerobic yeast, the enhancement of cellular growth is expected by raising the oxygen mass transfer (Magdouli et al. 2018). Nevertheless, the mechanical stress promoted by high agitation rates or oxidative stress resulting from the high dissolved oxygen concentration may inhibit cellular metabolism (Alonso et al. 2005). Though no significant differences were observed on growth rate

of *Y. lipolytica* NCYC 2904 (0.29 h^{-1} ± 0.07 h^{-1}) and W29 strain (0.23 h^{-1} ± 0.01 h^{-1}) at higher k_La conditions, the final biomass of NCYC 2904 cultures was 40 % higher than that of W29 (Figure 3.6A and 3.6B). As already observed in Erlenmeyer flask experiments, also in bioreactor experiments NCYC 2904 strain demonstrated an intrinsic ability to grow better than W29 strain on VFAs, regardless of oxygenation conditions. Notwithstanding the higher growth, total VFAs uptake rate of NCYC 2904 strain was similar to W29 strain (Table 3.5). The VFAs assimilation was clearly affected by oxygenation conditions and a 95 % improvement was obtained by increasing k_La from 22 h^{-1} to 87 h^{-1} or to 125 h^{-1}. Whereas a complete consumption of VFAs was observed after 24 h for both strains at higher k_La conditions, a significant amount of unconsumed VFAs remained in the culture medium in experiments carried out at k_La of 22 h^{-1} (Figure 3.6C and 3.6D). Comparatively to batch cultures in Erlenmeyer flask experiments (VFAs + glucose, Table 3.1), VFAs uptake rate obtained at k_La of 125 h^{-1} was 25 % higher ($p < 0.05$), since oxygenation of culture medium leads to a metabolically more active cells, not limited by oxygen availability.

Table 3.5 - Experimental values of volumetric oxygen transfer coefficient (k_La), biomass yield ($Y_{X/S}$), maximum lipids yield ($Y_{L/S}$), total VFAs uptake rate (R_{VFAs}), maximum lipids content and maximum lipids concentration obtained in *Y. lipolytica* W29 and *Y. lipolytic*a NCYC 2904 batch cultures carried out in a STR bioreactor with a mixture of VFAs (2 $g{\cdot}L^{-1}$ acetate, 2 $g{\cdot}L^{-1}$ propionate and 2 $g{\cdot}L^{-1}$ butyrate) and glucose (20 $g{\cdot}L^{-1}$). Data are average ± standard deviation for two independent replicates. Values followed by the same letter do not present statistically significant differences ($p \geq 0.05$).

Strain	k_La (h^{-1})	$Y_{X/S}$ ($g{\cdot}g^{-1}$)	$Y_{L/S}$* ($g{\cdot}g^{-1}$)	R_{VFAs} ($g{\cdot}L^{-1}{\cdot}h^{-1}$)	Lipids content* (%, w/w)	Lipids concentration* ($g{\cdot}L^{-1}$)
W29	22	0.17 ± 0.03 ^{a}	0.04 ± 0.00 ^{a}	0.013 ± 0.001 ^{a}	7.5 ± 0.1 ^{a}	0.203 ± 0.003 ^{a}
	87	0.3 ± 0.1 ^{ab}	0.054 ± 0.006 ^{ab}	0.27 ± 0.4 ^{b}	11.5 ± 0.5 ^{b}	1.3 ± 0.2 ^{b}
	125	0.36 ± 0.03 ^{abc}	0.034 ± 0.006 ^{a}	0.28 ± 0.01 ^{b}	9.3 ± 0.3 ^{c}	1.0 ± 0.2 ^{b}
NCYC 2904	22	0.6± 0.1 ^{c}	0.09 ± 0.02 ^{bc}	0.058 ± 0.001 ^{a}	12.2 ± 0.8 ^{bd}	1.3 ± 0.2 ^{b}
	87	0.56 ± 0.03 ^{bc}	0.131 ± 0.005 ^{d}	0.264 ± 0.006 ^{b}	21.8 ± 0.3 ^{f}	3.60 ± 0.09 ^{c}
	125	0.54 ± 0.07 ^{bc}	0.10 ± 0.02 ^{c}	0.242 ± 0.005 ^{b}	13 ± 0.1 ^{d}	2.5 ± 0.7 ^{d}

*Values obtained after 48 h and 72 h of cultivation for *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904, respectively. $Y_{X/S}$ was calculated at the end of growth (72 h); R_{VFAs} was calculated in the first 24 h for experiments at k_La of 87 h^{-1} and 125 h^{-1}, and 72 h for k_La of 22 h^{-1}.

The effect of dissolved oxygen on lipids accumulation by *Y. lipolytica* is not consensual and no information regarding its influence on lipids production by *Y. lipolytica* from VFAs is available. In this study, and regardless of yeast strain, a clearly positive effect was observed on lipids production by increasing k_La to 87 h^{-1}. Approximately 7- and 3-fold improvement on microbial lipids concentration was attained for *Y. lipolytica* W29 and NCYC 2904 cultures, respectively (Table 3.5). According to Bellou et al. (2014), lipid synthesis is upregulated under high dissolved oxygen concentrations as result of the upregulation of enzymes involved in lipids synthesis (ATP-citrate lyase and malic enzyme). However, the further increase of oxygenation led to a considerable reduction on lipids synthesis by *Y. lipolytica* NCYC 2904. It was already reported that, in some cases, high oxygen concentration during lipids biosynthesis drives the yeast metabolism toward the production of lipid-free biomass, instead of lipids synthesis (Papanikolaou et al., 2007). It is worth to notice that there was no improvement in lipids production by *Y. lipolytica* W29 in bioreactor experiments comparatively with flask experiments, while lipids synthesis by *Y. lipolytica* NCYC 2904 were 40 % higher in this system. Unlike to *Y. lipolytica* NCYC 2904, that accumulated more intracellular lipids in batch cultures in a STR bioreactor, batch cultures may be not the most effective for lipids accumulation by *Y. lipolytica* W29. Among the experimental conditions studied, the highest lipids productivity was obtained in the experiments in which dissolved oxygen concentration remained above 70 % in stationary growth phase (k_La of 87 h^{-1}), that corresponds to the lipogenic phase in hydrophilic substrates. The increase of lipids synthesis by *Y. lipolytica* strains using highly aerated cultures was reported for glucose (Tai and Stephanopoulos 2013; Bellou et al. 2014) and crude glycerol (Rakicka et al. 2015). By contrast, some authors demonstrated that lipids production by *Y. lipolytica* from crude glycerol (Magdouli et al. 2018), waste cooking oils (Lopes et al., 2019) and pork lard (Lopes et al., 2018) was higher under low dissolved oxygen concentrations. Other microbial species, such as *Rhodotorula glutinis* growing in glucose (Yen and Zhang 2011) and *C. albidus* growing in VFAs (Fei et al. 2011b) enhanced their intracellular lipids content in conditions of poor oxygenation.

The results obtained during this study show that k_La is an important factor on microbial lipids production by *Y. lipolytica* strains from VFAs and higher lipids synthesis from VFAs is reached at k_La of 87 h^{-1}.

Lipids accumulated by *Y. lipolytica* strains in bioreactor was mainly composed by oleic acid (21 % - 56 %), followed by linoleic (13 % - 26 %) and palmitic (11 % - 30 %) acids (Table 3.6). Interestingly, at low k_La condition, *Y. lipolytica* W29 accumulated more saturated fatty acids than unsaturated one, which is not very common in this yeast. Furthermore, at low oxygenation conditions, margaric acid was not synthetized by both strains since propionate was not assimilated. It seems that there is a relation between oxygenation conditions and fatty acids composition, which may be interest to produce tailor-made lipids with specific

fatty acids composition. For *Y. lipolytica* W29, the unsaturated fraction increased with k_La values, attaining the highest unsaturated fraction (> 70 %) at k_La of 125 h^{-1}. Lopes et al. (2018) also concluded that the unsaturated fraction of microbial lipids accumulated by *Y. lipolytica* W29 from pork lard was high in highly aerated cultures. Although the unsaturated fraction of microbial lipids synthesized by NCYC 2904 decreased with k_La increase, its composition exceeds 77 %. Notwithstanding the differences in fatty acid profiles of lipids accumulated by the two *Y. lipolytica* strains, composition of fatty acids is similar to vegetable oils (rapeseed oil), with higher content in oleic and linoleic acids (Issariyakul and Dalai 2014), suggesting that microbial lipids produced by *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 from VFAs have great potential to be used for biodiesel production.

Table 3.6 - Fatty acids composition of microbial lipids produced by *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 under different oxygenation conditions in STR bioreactor. Data are average ± standard deviation for two independent replicates.

Strain	k_La (h^{-1})	Relative fatty acid content (%)					
		C16:0	C16:1	C17:0	C18:0	C18:1	C18:2
W29	22	30 ± 1	3.1 ± 0.2	-	21.8 ± 0.8	21.0 ± 0.9	25.8 ± 0.0
	87	15 ± 1	7.6 ± 0.9	9.2 ± 0.6	8 ± 2	38 ± 2	23 ± 2
	125	16 ± 2	8.7 ± 0.4	5 ± 1	6.3 ± 0.9	44.0 ± 0.8	19.6 ± 1.6
NCYC 2904	22	11.4 ± 0.5	7.7 ± 0.4	-	8.6 ± 0.3	56.3 ± 0.1	16.7 ± 0.6
	87	12.5 ± 0.5	12.4 ± 0.1	5.2 ± 0.2	4.9 ± 0.6	52 ± 1	13 ± 1
	125	12.2 ± 0.4	11.2 ± 0.5	4.7 ± 0.3	4.9 ± 0.6	50 ± 1	16 ± 1

3.4. Conclusions

In this study, it was shown the ability of *Y. lipolytica* strains to grow on VFAs (individually or mixed with addition of glucose or glycerol) and accumulate lipids intracellularly. The highest microbial lipids concentration reached by *Y. lipolytica* W29 (1.42 $g{\cdot}L^{-1}$) and NCYC 2904 strain (2.6 $g{\cdot}L^{-1}$) in batch cultures was obtained in experiments carried out with a mixture of VFAs supplemented with glucose (20 $g{\cdot}L^{-1}$). Moreover, the addition of co-substrates, like glucose or glycerol, suggest that the viability of this process may be improved by using simple sugars derived from agro-food wastes hydrolysis and food processing by-products (e.g., molasses) and with others industrial by-products such as crude glycerol from biodiesel production. In two-stage batch experiments, *Y. lipolytica* NCYC 2904 accumulated 34 % more intracellular

lipids than W29 strain with the addition of 18 $g \cdot L^{-1}$ of mixed VFAs after yeast growth on glucose. In lab-scale stirred bioreactor, culture oxygenation is an important factor affecting VFAs uptake rate and microbial lipids production. Whereas the increase of k_La had no significant differences on yeast growth, the total VFAs uptake rate was maximal at k_La of 87 h^{-1}. The highest lipids synthesis was also reached in experiments conducted at k_La of 87 h^{-1}. The fatty acids composition similar to vegetable oils – higher content in oleic and linoleic acids – found in lipids accumulated by both *Y. lipolytica* strains, demonstrated its great potential to be used as feedstock in biodiesel industry. The results presented herein provide valuable information for using VFAs as substrate for lipids production by oleaginous yeasts. The bioconversion of VFAs, which are generated during acidogenesis of organic municipal solid wastes, by *Y. lipolytica* can be an important step in the valorization of these residues. The exploitation of oleaginous yeasts as biorefinery platform to obtain valuable compounds (microbial lipids) from low-cost substrates (VFAs produced from biodegradable municipal wastes) is an interesting strategy inserted in the circular economy concept.

CHAPTER 4

Enhancing microbial lipids production by *Yarrowia lipolytica* W29 from volatile fatty acids: Two-stage batch strategies

This chapter is based on the following original research article:

Pereira, A. S., Belo, I., Lopes, M. (2022) Enhancing microbial lipids synthesis for biodiesel production by *Y. lipolytica* W29 from volatile fatty acids: two-stage batch strategies. Applied Sciences, 12 (17): 8614. https://doi.org/10.3390/app12178614

4.1. Introduction

Microbial lipids are under the spotlight during the last decades owing to their potential application as food supplements and nutraceuticals – polyunsaturated fatty acids (PUFA), such as those belonging to the omega-3 and omega-6 lipids – or as a renewable energy source for the biodiesel industry – saturated (SFA) and monounsaturated (MUFA) fatty acids (Patel et al. 2020).

Oleaginous yeasts are able to accumulate more than 20 % (w/w) of their dry weight as intracellular lipids, mainly in the form of triacylglycerols (TAG). Comparatively to other oleaginous microorganisms (e.g., filamentous fungi or microalgae) or vegetable crops, yeasts have many attractive characteristics including high biomass and lipids productivity, utilization of several substrates, less seasonal or climate dependence, short life cycle, higher metal ion tolerance and the relative ease of culturing (Vasconcelos et al. 2019; Bao et al. 2021). In particular, the non-conventional yeast *Yarrowia lipolytica* has been considered a model yeast for microbial lipids production, owing to its superior capacity for lipids accumulation and ability to use various and cheap raw materials, such as wastes and by-products of the agro-industrial sector (Lopes et al. 2022). Lipids accumulated by *Y. lipolytica* have, in general, a similar fatty acid composition and energy value to common vegetable oils, making them a promising feedstock for biodiesel production (Patel et al. 2020).

Biodiesel is a non-toxic, sulfur-free, renewable, and biodegradable fuel, and its combustion emits fewer greenhouse gases than petroleum diesel (Zhang et al. 2021). Biodiesel is mainly obtained from vegetable oils, but the limited supply and high cost of these feedstocks make its production not economically viable (Dharmaraja et al. 2019). Biodiesel derived from *Y. lipolytica* lipids is a promising technology and does not compete with arable land for food production as occurred with crop-based oil resources used as raw material for biodiesel production. However, the high costs of carbon and nitrogen sources, the amount of lipids accumulated per unit of cellular mass, and the post-processing costs associated with oil extraction from cells are still challenges for the large-scale production of microbial lipids (Jones et al. 2019). In this regard, the use of abundant, renewable, and low-value raw materials as substrates for microbial lipids production decreases the overall costs and enables the development of a more sustainable and affordable process within the circular bioeconomy assumptions.

Volatile fatty acids (VFAs), generated as intermediates during anaerobic fermentation of organic wastes, have been appointed as a renewable and low-cost feedstock for microbial lipids production by *Yarrowia* species (Gao et al., 2017; Llamas et al., 2020; Pereira et al., 2021). Regardless of the substrate and anaerobic fermentation conditions, VFAs comprise short-chain organic acids (C2 – C6), in particular acetic (30 % - 80 %), propionic (5 % - 32 %), and butyric (11 - 30 %) acids (Patel et al., 2021; Pereira et al.,

2022). Comparatively to sugar-based substrates, these organic acids have higher theoretical conversion yields and shorter metabolic pathways to intracellular lipids (Gong et al. 2016). The main strategies reported so far studied the conversion of VFAs to microbial lipids in batch cultures (Gao et al., 2017; Llamas et al., 2020; Patel et al., 2021; Pereira et al., 2021). Previous works demonstrated that the two-stage batch strategy (growth phase in glucose, followed by the addition of VFA for lipid synthesis) enhances microbial lipids production by *Y. lipolytica* (Pereira et al. 2021). Thus, this study aims to evaluate the ability of *Y. lipolytica* W29 to synthesize intracellular lipids from a VFAs mixture (acetate, propionate, and butyrate) during the lipogenic phase after yeast growth in glucose or glycerol. For the first time, a two-stage batch strategy with 3 pulses of 6 $g \cdot L^{-1}$ VFAs mixture was tested. *Yarrowia lipolytica* W29 was able to assimilate 18 $g \cdot L^{-1}$ VFAs without inhibition of VFAs uptake rate and cellular growth, producing higher amounts of microbial lipids. The estimation of the properties of biodiesel produced from *Y. lipolytica* W29 lipids indicates that these lipids have a high value for the biodiesel industry. The results obtained are quite promising for the development of a more economical and sustainable bioprocess for the production of microbial lipids by a *Y. lipolytica* wild strain from VFAs, particularly in future works using organic wastes-derived VFAs.

4.2. Materials and Methods

4.2.1. Yeast strain and inoculum preparation

Yarrowia lipolytica W29 was preserved in cryo-stocks at - 80 °C and inoculum was prepared as described in chapter 3.2.1.

4.2.2. Microbial lipids production in two-stage batch cultures

Two-stage batch cultures were firstly carried out in 500-mL baffled Erlenmeyer flasks filled with 200 mL of medium. *Yarrowia lipolytica* W29 cells grew for 24 h in glucose or glycerol medium (20 $g \cdot L^{-1}$ glucose or glycerol, 0.5 $g \cdot L^{-1}$ yeast extract, 1.7 $g \cdot L^{-1}$ YNB without amino acids and without ammonium sulfate, and ammonium sulfate to obtain an initial C/N mass ratio of 75) – growth phase. This phase was followed by the addition of (a) one pulse of 6 $g \cdot L^{-1}$ VFAs mixture (2 $g \cdot L^{-1}$ propionate, 2 $g \cdot L^{-1}$ butyrate, and 2 $g \cdot L^{-1}$ acetate); or (b) 3 pulses of 6 $g \cdot L^{-1}$ VFAs mixture (24 h, 48 h, 72 h) – lipogenic phase. Additionally, an experiment with a single pulse of 18 $g \cdot L^{-1}$ VFAs mixture (6 $g \cdot L^{-1}$ propionate, 6 $g \cdot L^{-1}$ butyrate and 6 $g \cdot L^{-1}$ acetate) after yeast growth in glucose for 24 h was also performed. All experiments were carried out at an initial pH of 6, at 27 °C and 170 rpm in an orbital incubator.

4.2.3. Analytical methods

Samples were taken at appropriate intervals for the quantification of biomass, glucose, glycerol, and VFAs concentration, intracellular lipids content, and long-chain fatty acids (LCFA) composition. The analysis of samples was performed as described in the chapter 3.2.6.

4.2.4. Biodiesel properties

The principal properties of biodiesel (cetane number, higher heating value, cloud point, oxidative stability, iodine value, cold filter plugging point, degree of unsaturation, kinematic viscosity, density, pour point, and saponification value) were estimated by considering the fatty acids composition of microbial lipids, using the software BiodieselAnalyzer© Ver. 2.2 (available on "http:// www. brteam.ir/biodieselanalyzer") (Talebi et al. 2014).

4.2.5. Statistical analysis

One-way analysis of variance (ANOVA) was used for the statistical analysis of the data as described in chapter 3.2.8.

4.3. Results and Discussion

4.3.1. Microbial lipids production in two-stage batch cultures

The amount of microbial lipids produced in batch cultures is usually low owing to the difficulties of optimizing the culture and operational conditions for simultaneous cellular growth (nitrogen-rich media) and lipids synthesis (nitrogen-limited media) (Karamerouand Webb, 2019). Two-stage batch cultures (TSC), an approach that encompasses two distinct phases - the growth phase (1st phase), followed by the lipid synthesis in the lipogenic phase (2nd phase) - was proved to be advantageous for increasing lipids production by *Y. lipolytica* MUCL 28849 (Fontanille et al. 2012), *Cryptococcus curvatus* (Chatterjeeand Mohan, 2020) and and *Apiotrichum porosum* (Qian et al. 2021) (Table 4.1). Thus, in an attempt to achieve high lipids concentration in *Y. lipolytica* W29 cultures, several experiments were carried out in which the cell proliferation phase in glucose or glycerol was followed by the lipogenic phase in VFAs.

Table 4.1 - Comparison of biomass and microbial lipids concentration obtained by different microbial species from VFAs in two-stage batch and two-stage fed-batch cultures.

Microorganism	Operation Mode	Feedstock	Biomass ($g \cdot L^{-1}$)	Lipids ($g \cdot L^{-1}$)	Reference
Y. lipolytica MUCL 28849	Two-stage fed-batch	40 $g \cdot L^{-1}$ of glucose + 5 $g \cdot L^{-1}$ of VFAs	41	16.5	Fontanille et al. (2012)
		40 $g \cdot L^{-1}$ of glycerol + 5 $g \cdot L^{-1}$ of VFAs	41	14.2	
Cryptococcus curvatus MTCC 2698	Two-stage batch	40 $g \cdot L^{-1}$ of glucose + 5 $g \cdot L^{-1}$ of VFAs	4.6	1.06	Chatterjee and Mohan (2020)
Apiotrichum porosum DSM 27194	Two-stage fed-batch	15 $g \cdot L^{-1}$ of glucose + 5 $g \cdot L^{-1}$ of VFAs	26.5	9.6	Qian et al. (2021)

Yarrowia lipolytica W29 grew for 24 h in glucose or glycerol (growth phase) to obtain a higher cell density, followed by the addition of one pulse of 6 $g \cdot L^{-1}$ VFA mixture (lipogenic phase) to produce microbial lipids. During the first phase, yeast growth was similar in both substrates, no lag phase was observed and the biomass concentration obtained after 24 h of cultivation was similar (Figure 4.1A and 4.1B). Glucose and glycerol were completely consumed after 24 h with a similar uptake rate (Table 4.2). In the lipogenic phase, *Y. lipolytica* cells continued to grow due to the addition of VFAs that were also used for biomass proliferation, but with a lower specific growth rate. The stationary growth phase was reached at 48 h in both experiments – coinciding with the VFA consumption – and the final biomass concentration and the global biomass yield were statistically equal ($p \geq 0.05$). VFAs were consumed by yeast cells with different uptake rates, depending on the substrate used in the growth phase (Table 4.2). However, in both conditions, it was noted a preference of yeast for acetate, being completely consumed 12 h after the VFAs addition. Butyrate took 24 h to be assimilated in both experiments, whereas butyrate was consumed after 24 h and 30 h of VFAs addition to glucose (Figure 4.1A) and glycerol (Figure 4.1B) media, respectively.

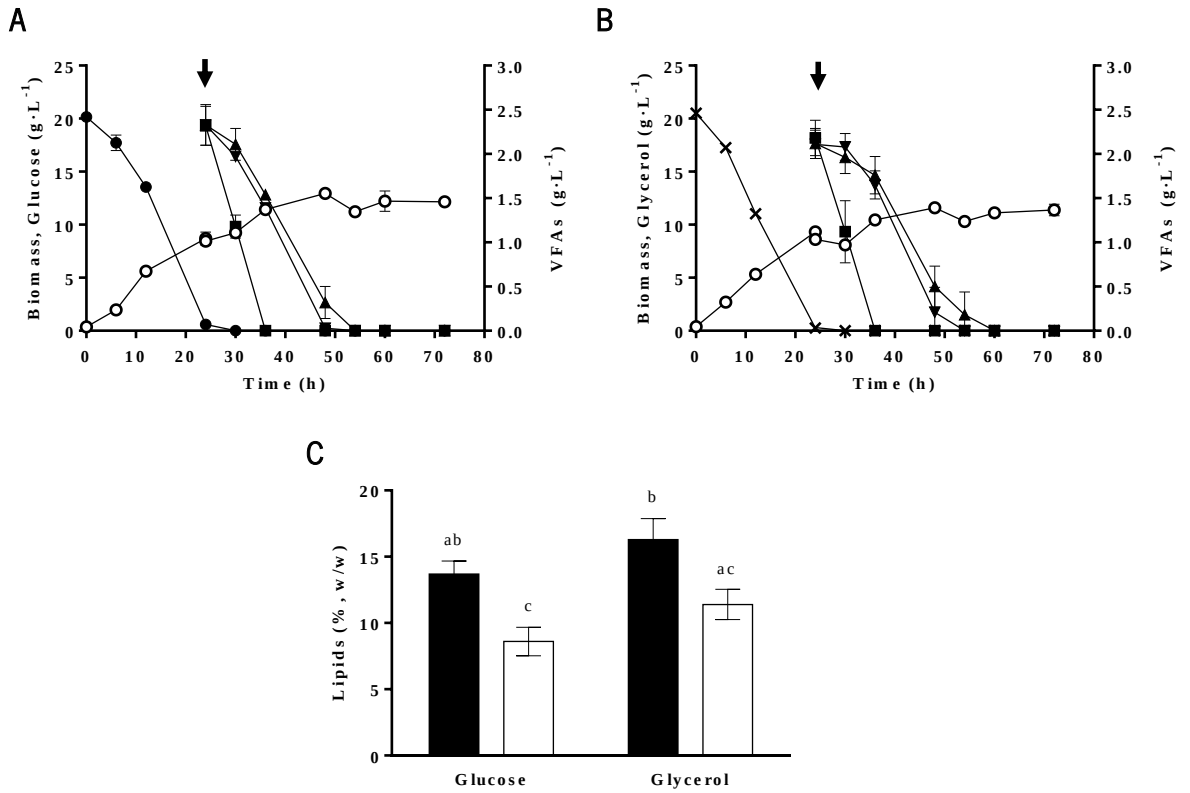

Figure 4.1 - Time course of cellular growth (○), VFAs (■, acetate; ▲, propionate; ▼, butyrate), glucose (●), and glycerol (×) concentration obtained in two-stage batch cultures of *Y. lipolytica* W29. After 24 h of growth in glucose (A) or glycerol (B), a mixture of 6 g·L^{-1} VFAs (2 g·L^{-1} acetate, 2 g·L^{-1} propionate, and 2 g·L^{-1} butyrate) was added to the culture medium. Microbial lipids content of *Y. lipolytica* cells at 48 h (black bars) and 72 h (white bars) of cultivation (C). The error bars represent the standard deviation of two independent replicates.

The lipids content of *Y. lipolytica* W29 cells was not affected by the substrate used during the growth phase. Regardless of the substrate used in the growth phase, yeast cells accumulated higher amounts of lipids at 48 h of cultivation (24 h after the VFAs addition), decreasing 1.5 times at 72 h (48 h after the VFAs addition) (Figure 4.1C). In all conditions, the maximum lipids production occurred at the end of the exponential growth phase, and utilization of intracellular lipids for metabolic activities was observed in the stationary phase owing to the complete depletion of the substrates (Figures 4.1A and 4.1B).

Table 4.2 - Values of global biomass yield ($Y_{X/S}$), maximum lipids concentration and substrate (glucose or glycerol) uptake rate, and VFAs (R_{VFAs}) obtained in *Y. lipolytica* W29 two-stage batch cultures. A mixture of 6 g·L^{-1} VFAs (2 g·L^{-1} acetate, 2 g·L^{-1} propionate, and 2 g·L^{-1} butyrate) was added in each pulse. Data are average ± standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences ($p \geq 0.05$).

Substrate (Growth Phase)	Pulses	$Y_{X/S}$ (g·g^{-1})	Lipids (g·L^{-1})	R_s* (g·L^{-1}·h^{-1})	R_{VFAs}** (g·L^{-1}·h^{-1})
Glucose	1	0.43 ± 0.01 ^{a}	1.8 ± 0.1 ^{a}	0.84 ± 0.01 ^{a}	0.28 ± 0.01 ^{a}
	3	0.35 ± 0.01 ^{b}	2.9 ± 0.2 ^{b}	0.83 ± 0.01 ^{b}	0.25 ± 0.01 ^{ab} 0.20 ± 0.002 ^{b} 0.20 ± 0.02 ^{b}
Glycerol	1	0.41 ± 0.01 ^{ac}	1.9 ± 0.3 ^{a}	0.87 ± 0.001 ^{a}	0.22 ± 0.02 ^{b}
	3	0.39 ± 0.004 ^{bc}	2.9 ± 0.6 ^{b}	0.84 ± 0.01 ^{a}	0.25 ± 0.01 ^{ab} 0.28 ± 0.01 ^{a} 0.24 ± 0.02 ^{ab}

* Values correspond to the growth phase (first stage of cultivation). ** Values correspond to the lipogenic phase (second stage of cultivation). $Y_{X/S}$ was calculated at the end of the experiments considering glucose and VFAs as substrates. Maximum lipids concentrations were obtained after 24 h and 48 h of the addition of a single pulse and three pulses, respectively.

As VFAs were completely consumed 30 h after the addition and a lipid mobilization were observed in the lipogenic phase, two-stage batch cultures with 3 pulses of 6 g·L^{-1} VFAs mixture (24 h, 48 h, 72 h) were carried out as an attempt to prevent substrate depletion, lipids turnover and to boost lipids production. No enhancement in global biomass yield was attained in the experiments with 3 pulses compared to cultures with a single pulse (Table 4.2), but a higher biomass concentration was reached in glycerol medium after 3 pulses of VFAs (Figure 4.2B). *Yarrowia lipolytica* cells continued to grow after the addition of the first two pulses of VFAs, particularly in the experiments in which the growth phase was carried out with glycerol. However, yeast cells reached the stationary growth phase after the third pulse in both conditions, despite the almost total VFAs consumption (Figures 4.2A and 4.2B). Moreover, with the addition of two further pulses, VFAs were consumed at a similar uptake rate to that observed after the first pulse. These results suggest that VFAs were channelled towards cellular maintenance, lipids biosynthesis, or other metabolites instead of cell proliferation.

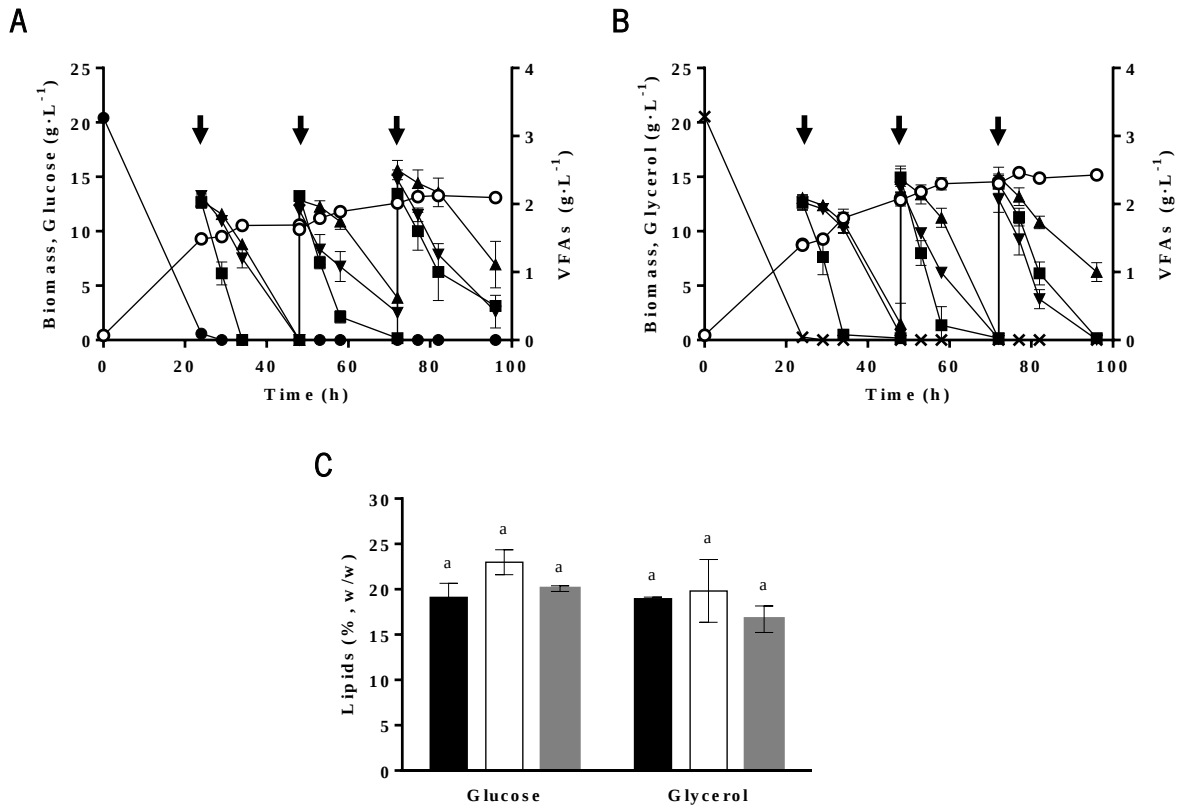

Figure 4.2 - Time course of cellular growth (○), VFAs (■, acetate; ▲, propionate; ▼, butyrate), glucose (●), and glycerol (×) concentration obtained in two-stage batch cultures of *Y. lipolytica* W29. After 24 h of growth in glucose (A) or glycerol (B), three pulses of a mixture of 6 g·L^{-1} VFAs (2 g·L^{-1} acetate, 2 g·L^{-1} propionate, and 2 g·L^{-1} butyrate) was added to the culture medium at 24 h, 48 h, and 72 h. Microbial lipids content of *Y. lipolytica* cells at 48 h (black bars), 72 h (white bars), and 96 h (grey bars) of cultivation (C). The error bars represent the standard deviation of two independent replicates. Bars with the same letter do not present statistically significant differences ($p \geq 0.05$).

The accumulation of microbial lipids occurred simultaneously with yeast growth and the lipids content was similar throughout the lipogenic phase, demonstrating that the cultures with 3 pulses of VFA were an effective strategy to prevent lipid turnover (Figure 4.2C). The addition of 3 pulses made it possible to maintain the VFAs concentration above zero during 96 h of cultivation (Figure 4.2), which contributed to preventing lipids turnover. Moreover, the maximum lipids content was reached in the stationary growth phase, indicating that the VFAs added were channelled towards lipids biosynthesis instead of biomass production. Furthermore, an approximately 2-fold improvement in lipids concentration was attained in cultures with 3 pulses, compared to those obtained in cultures with a single pulse (Table 4.2).

As *Y. lipolytica* demonstrated the ability to consume a total VFAs concentration of 18 $g \cdot L^{-1}$ (divided into 3 pulses), an additional experiment was carried out with a 24 h first stage of yeast growth in glucose followed by the addition of a single pulse of 18 $g \cdot L^{-1}$ VFAs (mixtures of acetate, butyrate, and propionate with 6 $g \cdot L^{-1}$ of each one) in the second stage. From an operational point of view and, considering the industrial production of lipids, a single addition would be more advantageous. Moreover, other *Y. lipolytica* strains have already shown the ability to assimilate high VFAs amounts without inhibitory effects (Pereira et al. 2022b). *Yarrowia lipolytica* was able to consume 18 $g \cdot L^{-1}$ VFAs without inhibitory effects on cellular growth (Figure 4.3A) and VFAs uptake rate. No statistical differences were observed in the VFAs uptake rate between experiments with a pulse of 18 $g \cdot L^{-1}$ VFA (0.19 $g \cdot L^{-1} \cdot h^{-1} \pm 0.002$ $g \cdot L^{-1} \cdot h^{-1}$) and with a pulse of 6 $g \cdot L^{-1}$ VFAs. As occurred in the previous experiments, yeast cells demonstrated a preference for acetate, being totally consumed 48 h after the pulse. By contrast, butyrate took 72 h to be completely assimilated and a small amount of propionate (1.5 $g \cdot L^{-1}$) remained in the medium at the end of the cultivation. The global biomass yield in these experiments (0.28 $g \cdot g^{-1} \pm 0.01$ $g \cdot g^{-1}$) was lower than those obtained with a single pulse of 6 $g \cdot L^{-1}$ VFA and three pulses of 6 $g \cdot L^{-1}$ VFA each, suggesting that VFAs were more directed for lipids synthesis than for cellular growth. In fact, maximum lipids content (Figure 4.3B) was higher ($p < 0.05$) than that attained in the previous two-stage batch culture strategies. Moreover, no significant mobilization of lipids was observed during the cultivation, except for the last point (96 h) owing to the depletion of VFAs. The maximum lipids concentration was similar after the addition of one pulse (18 $g \cdot L^{-1}$) or three pulses of VFAs, and no differences in the maximum lipids productivity were observed between these two-stage batch strategies. Thus, a two-stage batch culture with a single pulse of 18 $g \cdot L^{-1}$ VFAs is an efficient strategy to improve lipids production without significant lipids mobilization in the lipogenic phase. By contrast, this was the experiment where lower biomass production was reached. In this condition, a higher amount of VFAs was supplemented at once, resulting in a higher carbon/nitrogen (C/N) ratio and favoring lipids production. According to the literature, in a *de novo* lipid biosynthesis pathway - as occurs from glucose and acetate - high C/N ratios favor lipids synthesis instead of yeast cell proliferation. This metabolic route is generally induced by the limitation of nitrogen, leading to a decrease in yeast growth, since this is an essential nutrient for nucleic acid and protein synthesis. Yet, the carbon sources continue to be assimilated and are channelled for lipids biosynthesis (Karamerou and Webb, 2019; Papanikolaou and Aggelis, 2011). It has been reported that the biosynthesis of microbial lipids by *Y. lipolytica* from butyrate and propionate is an *ex novo* metabolic pathway (Morales-Palomo et al. 2022), which is independent of the C/N ratio (Papanikolaou and Aggelis, 2011). However, in this study, a clear preference for acetate - which is uptaken via the *de novo* pathway - was observed.

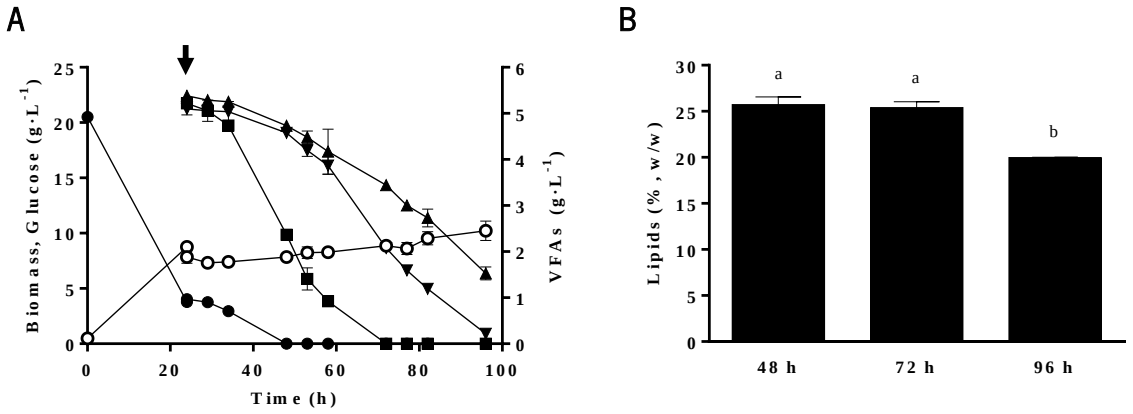

Figure 4.3 - Time course of cellular growth (○), VFAs (■, acetate; ▲, propionate; ▼, butyrate), and glucose (●) concentration obtained in two-stage batch cultures of *Y. lipolytica* W29 (A). After 24 h of growth in glucose, one single pulse of a mixture of 18 g·L^{-1} VFAs was added to the culture medium. Microbial lipids content of *Y. lipolytica* cells obtained at 48 h, 72 h, and 96 h of cultivation (B). The error bars represent the standard deviation of two independent replicates. Bars with the same letter do not present statistically significant differences ($p \geq 0.05$).

Microbial lipids produced in two-stage batch cultures of *Y. lipolytica* W29 (growth phase in glucose followed by the addition of 18 g·L^{-1} VFAs) were mainly composed of oleic acid (54 % ± 1 %), followed by linoleic (15.1 % ± 0.2 %), palmitic (11.9 % ± 0.4 %), stearic (7.7 % ± 1.0 %) and palmitoleic (5.5 % ± 0.2 %) acids. Due to the presence of propionate in the lipogenic phase (Pereira et al. 2021), odd-chain fatty acids as heptadecenoic (3.8 % ± 0.4 %) and margaric (2.2 % ± 0.3 %) acids were also produced by Y. lipolytica. Lipids produced in these conditions have a significantly higher unsaturated fraction (> 78 %, $p < 0.05$) than saturated one. The high resemblance between the composition of *Y. lipolytica* lipids and vegetable oils makes these bio-oils a potential substitute for vegetable oils as feedstock for biodiesel production. Moreover, the odd-chain fatty acids (heptadecenoic and margaric acids) synthesized in these conditions will improve the fuel properties of biodiesel produced from *Y. lipolytica* lipids (Park et al. 2018). Other *Y. lipolytica* strains, growing in VFAs (Gao et al. 2020; Pereira et al. 2021), glycerol (Poli et al. 2014), or in hydrophobic substrates - waste cooking oils (Lopes et al. 2019) and lard (Lopes et al. 2018) - accumulated preferentially unsaturated fatty acids (oleic and linoleic acids). This behavior suggests that lipogenic features of *Y. lipolytica* prevail over the type of substrate.

4.3.2. Biodiesel properties obtained from *Y. lipolytica* W29 lipids

Based on the fatty acid composition of *Y. lipolytica* W29 lipids, synthesized in two-stage batch cultures with a pulse of 18 $g \cdot L^{-1}$ VFA, several properties that assess the quality of biodiesel were estimated using the BiodieselAnalyzer© software. The main parameters predicted - density, kinematic viscosity, cetane number, oxidative stability, and iodine value - are within the ranges of the EU biodiesel standard EN 14214 (Table 4.3). Moreover, some of the parameters, namely density, kinematic viscosity, cetane number, higher heating value, oxidative stability, and iodine value are comparable with those of biodiesel produced from lipids of other *Y. lipolytica* strains growing in nondetoxified liquid wheat straw hydrolysate (Patel et al. 2017).

Table 4.3 - Biodiesel properties obtained from *Y. lipolytica* W29 lipids estimated by the BiodieselAnalyzer© software, and comparison with other *Y. lipolytica* strains and the EU biodiesel standard EN 14214.

Properties	*Y. lipolytica* W29 (This Study)	*Y. lipolytica* (Patel et al. 2017)	EU Biodiesel Standard EN 14214
Density ($kg \cdot m^{-3}$)	862	860	860–900
Kinematic viscosity ($mm^2 \cdot s^{-1}$)	3.7	4.5	3.5–5.0
Cetane number	56	58	47 min
Higher heating value ($MJ \cdot kg^{-1}$)	38	42	NR
Cloud point (°C)	1.3	NR	NR
Oxidative Stability (h)	10.4	8.5	6 min
Iodine value (mg I_2/100 g)	81.2	80	120 max
Degree of unsaturation	89.5	NR	NR
Cold filter plugging point (°C)	−0.6	−10	NR *
Pour Point (°C)	−5.4	NR	NR
Saponification value ($mg \cdot g^{-1}$)	194.6	160	NR

NR not reported; * CFPP limits depend on geography and time of the year; min, minimum; max, maximum; h, hours

These properties are affected by the fatty acid composition of oils used in the transesterification reactions, namely chain length, unsaturation degree, and number and position of double bonds. High saturated fatty acids, for example, avoid the auto-oxidation of biofuel and increase its shelf life, while the amount of unsaturated fatty acids has an effect on biodiesel's cold flow plugging properties (Patel et al. 2017). The cetane number of diesel fuel is an index of the ignition quality (ignition delay characteristics) and defines

the quality of the combustion process in a diesel engine, affecting the stability, noise, and CO emissions. A low cetane number (long ignition delay, particularly in cold environments) implies that the majority of injected biodiesel burns explosively, resulting in loud diesel combustion noise, white smoke, and hydrocarbon emissions. By contrast, a high cetane number indicates a shorter ignition delay and a more uniform and complete burning of injected fuel, leading to better exhaust air quality. However, a cetane number higher than 65 results in excessive heating of the injector and in a reduction of biofuel efficiency (Patel et al. 2017; Yaşar 2020). The estimated cetane number for biodiesel produced from *Y. lipolytica* W29 lipids (56) meets this criterion and is comparable to the cetane number of biodiesel obtained from different vegetable oils (Yaşar 2020). The high heating value of biodiesel describes the heating energy released during the combustion, i.e., the efficiency of the biofuel. The value predicted herein (38 $MJ \cdot kg^{-1}$) is in the range of others found in the literature for biodiesel produced from pure and used vegetable oils, and lipids of *Y. lipolytica* strains grown in wheat straw hydrolysate (Fassinou et al. 2010; Nambou et al. 2014). The cold filter plugging point defines the lowest temperature, expressed in degrees Celsius (°C), at which a given volume of biodiesel easily flows through a standardized filtration device in a specific time when cooled under certain conditions (Patel et al. 2017). Though there are no specific recommendations for the cold filter plugging point, the lower this value is, the better the cold flow properties of the biofuel. This is especially important in cold temperate countries since biodiesel with a high cold filter plugging point will clog the fuel line and filters more easily. The value of the cold filter plugging point estimated in this work (–0.6 °C) is lower than the value obtained using vegetable oils (Yaşar 2020) and lipids of *Trichosporon* sp. (Brar et al. 2017), and higher than those obtained with lipids of other *Y. lipolytica* strains (Patel et al. 2017). The degree of unsaturation and number of double bonds in fatty acids of oils has negative effects on the oxidative stability and biodiesel shelf life, since oxidatively unstable biodiesel leads to the formation of gums and sedimentation, decreasing the engine performance (Patel et al. 2017). The oxidative stability obtained herein (10.4 h) is similar to that of biodiesel produced using lipids of different *Y. lipolytica* strain growing and detoxified and non-detoxified liquid wheat straw hydrolysate (Patel et al. 2017). The kinematic viscosity of biodiesel, related to the ability of flowing, increases at low temperatures and affects the quantity and quality of injection and ignition. On the hand, lower values of kinematic viscosity result in fine particles of biodiesel with high speed and low mass (Patel et al. 2017). The value of kinematic viscosity of biodiesel obtained from microbial lipids of *Y. lipolytica* W29 was similar to that for *R. fluviale*, *R. toruloides,* and *Lipomyces starkeyi*, and higher than *Y. lipolytica* strains growing in detoxified and non-detoxified liquid wheat straw hydrolysate (Patel et al. 2017). Other estimated properties, such as iodine value and density fall within a narrow range of the EU biodiesel standard EN

14214 and are similar to those of biodiesel produced from common vegetable oils and other oleaginous yeasts (Patel et al. 2017; Yaşar 2020). Therefore, the intracellular lipids of *Y. lipolytica* W29 accumulated from VFAs in two-stage batch cultures have a high potential to be used as feedstock for high-quality biodiesel production. Yet, it is possible to make the process even more economically competitive by increasing the concentration of lipids produced (grams of lipids per liter of medium) if high-cell density cultures were attained (e.g., using a fed-batch mode for biomass production).

4.4. Conclusions

The results demonstrate the potential of *Y. lipolytica* W29 to grow and produce microbial lipids from a mixture of VFAs (acetate, propionate, and butyrate) in two-stage batch cultures - growth phase in glucose or glycerol, followed by the lipogenic phase in VFAs. A single pulse of 6 $g \cdot L^{-1}$ VFAs strategy led to the mobilization of microbial lipids synthesized owing to the complete consumption of VFAs after 48 h of cultivation. However, the addition of a higher amount of VFAs (3 pulses of 6 $g \cdot L^{-1}$ VFAs each or 1 pulse of 18 $g \cdot L^{-1}$ VFAs) avoided the complete depletion of VFAs for the duration of the experiments, increased the C/N ratio, boosted the production of microbial lipids, and prevented the lipid turnover. *Yarrowia lipolytica* lipids, rich in unsaturated fatty acids (particularly oleic acid) and odd-chain fatty acids (heptadecenoic and margaric acids), are a promising feedstock for the bioenergy industry. The biodiesel produced from these lipids meets the criteria of the EU biodiesel standard EN 14214, demonstrating the potential of *Y. lipolytica* W29 as a pivot in the production of feedstock for high-quality biofuels from low-cost substrates, according to the biorefinery and bioeconomy circular guidelines. The results described herein open new perspectives on the development of an integrated bioprocess, in which crude glycerol (a by-product of the biodiesel industry) or glucose-rich by-products (e.g., lignocellulosic biomass hydrolysates) could be the substrate for cellular growth, and organic wastes-derived VFAs (obtained by anaerobic fermentation) will be used for lipids synthesis.

CHAPTER 5

Microbial lipid production for biodiesel industry by *Yarrowia lipolytica* NCYC 2904 from volatile fatty acids: Two-stage batch culture

This chapter is based on the following original research article:

Pereira, A. S., Lopes, M.; Miranda, S. M.; Belo, I. (2022) Bio-oil production for biodiesel industry by *Yarrowia lipolytica* from volatile fatty acids in two-stage batch culture. Applied Microbiology and Biotechnology, 106 (8): 2869-2881. https://doi.org/10.1007/s00253-022-11900-7

5.1. Introduction

The global increase in energy demands, the depletion of fossil fuel reserves, climate change and environmental pollution drive for a continuously search of renewable and environmentally friendly energy sources. Biodiesel is a renewable energy and a promising alternative for replacement of conventional fossil fuels due to its biodegradable, non-toxic and energy efficiency properties. Currently, most of biodiesel is produced from vegetable oils (palm, soybean, rapeseed and sunflower), which are broadly used for food purposes, cosmetics, feed and oleochemical industries (Ghaly et al. 2010). The competition for crops, as well the dependence on land and climatic conditions, lead to a reduced availability of vegetable oils for biodiesel industry and to an increase of prices (Ghaly et al. 2010; Szczepańska et al. 2021). In recent years, microbial lipids produced by oleaginous microorganisms have received considerable attention as promising candidates for biodiesel production, since their composition is similar to that of vegetable oils (Patel et al. 2020). In particular oleaginous yeasts, that can accumulate more than 20 % (w/w) of their dry weight as intracellular lipids, have shown high lipids and biomass productivity, short life cycle and the relative ease of culturing compared to other oleaginous microorganisms (e.g., microalgae or filamentous fungi) or vegetable crops (Vasconcelos et al. 2019). However, microbial lipids production at large-scale is still hindered by the high cost of pure substrates, which usually accounts for about 80 % of the total production costs (Fei et al. 2011a). Thus, the use of low-cost, abundant and renewable feedstocks for microbial lipids production is a developing approach as it allows the decrease of overall process costs.

Approximately 931 million tons of food wastes (121 tons per capita) are generated worldwide each year (UNEP 2021) and its disposal without an adequate treatment (e.g., landfills) leads to serious environmental problems (Scherhaufer et al. 2018). Anaerobic fermentation is one of the most common alternative and sustainable technology for food waste treatment, allowing to convert these residues into volatile fatty acids (VFAs), which concentration may range from 4 $g \cdot L^{-1}$ to 40 $g \cdot L^{-1}$. Regardless of substrate and fermentation conditions, the most common VFAs generated during the anaerobic fermentation of food wastes are acetic (30 % – 80 %), propionic (5 % – 32 %) and butyric (11 % – 30 %) acids (Gameiro et al. 2016; Gao et al. 2017; Moretto et al. 2019a; Gao et al. 2020). The potential of VFAs as a less expensive carbon source for the cost-effective production of microbial lipids has been studied (Fontanille et al. 2012; Gao et al. 2017). Besides their cost-effective sources, VFAs have higher theoretical conversion efficiencies and shorter metabolic pathways to lipids compared with sugar-based carbon sources (Zheng et al. 2012; Gong et al. 2016). Most of the studies on lipids production by oleaginous yeasts have been performed with VFAs concentrations up to 15 $g \cdot L^{-1}$ (Rodrigues and Pais 2000; Fontanille et al. 2012;

Kolouchová et al. 2015; Gao et al. 2017) and some strategies have been explored to increase lipids production from VFAs, such as operation mode or addition of co-substrates (Fei et al. 2011a; Kolouchová et al. 2015; Pereira et al. 2021), but most of them were evaluated in Erlenmeyer flask and the lipids yield in these studies is still unsatisfactory. Our previous work (Pereira et al. 2021) demonstrated that intracellular lipids production by *Yarrowia lipolytica* was enhanced by the supplementation of a 6 $g \cdot L^{-1}$ VFAs mixture (acetate, propionate and butyrate) with glucose or glycerol in batch cultures. Moreover, the results obtained in Erlenmeyer flask experiments revealed the potential of employing two-stage batch cultures as a strategy to enhance lipids biosynthesis from VFAs. Unlike traditional batch cultivations, in which is difficult to achieve high productivities, since carbon sources are channeled simultaneously for yeast growth and lipids production, in two-stage cultivation approaches, cells proliferation (growth phase) and lipids synthesis (lipogenic phase) are separated to maximize the potential of each phase, enhancing both biomass and lipids production (Karamerou and Webb 2019).

This work firstly addresses the feasibility of using high VFAs mixture concentration (18 $g \cdot L^{-1}$), as sole carbon source or supplemented with glucose, for batch production of intracellular lipids by *Y. lipolytica* NCYC 2904 in a lab-scale stirred tank reactor (STR). Additionally, different two-stage batch cultures approaches (1^{st} stage – growth in glucose; 2^{nd} second stage – lipids production in 18 $g \cdot L^{-1}$ VFAs) were conducted in a lab-scale STR as an attempt to enhance lipids synthesis by *Y. lipolytica* NCYC 2904. Our previous studies showed that oxygenation conditions are key factors for lipids production by *Y. lipolytica* from VFAs in batch cultures (Pereira et al. 2021), but its effect on two-stage batch cultures was never evaluated. Thus, the effect of dissolved oxygen concentration during lipogenic phase on lipids synthesis was assessed, varying the agitation and aeration in 2^{nd} stage of cultivation.

5.2. Materials and Methods

5.2.1. Yeast strain and inoculum preparation

Yarrowia lipolytica NCYC 2904 was preserved in cryo-stocks at - 80 °C and inoculum was prepared as described in chapter 3.2.1.

5.2.2. Bioreactor experiments

Microbial lipids production by *Y. lipolytica* NCYC 2904 was carried out in a 5-L stirred tank bioreactor (STR) (BIOSTAT® B Plus, Sartorius) with an initial working volume of 2.5 L, at 27 °C and pH 6, maintained by the addition of HCl 2 M or NaOH 2 M. The dissolved oxygen concentration in culture medium was measured with an optical probe (InPro 6860i, Mettler Toledo, USA) coupled with the respective meter (Solaris Biotechnology SRL, Italy).

5.2.2.1. Batch cultures

Batch cultures for lipids production were carried out in STR lab-scale bioreactor at a specific air flow rate of 1 vvm and an agitation rate of 400 rpm. Three different production media were tested: (1) glucose medium composed by 20 $g \cdot L^{-1}$ glucose, 0.5 $g \cdot L^{-1}$ yeast extract, 1.7 $g \cdot L^{-1}$ YNB (without amino acids and without ammonium sulfate) and 0.5 $g \cdot L^{-1}$ ammonium sulfate; (2) VFAs-based medium composed by 18 $g \cdot L^{-1}$ VFAs (6 $g \cdot L^{-1}$ acetate, 6 $g \cdot L^{-1}$ propionate and 6 $g \cdot L^{-1}$ butyrate), 0.5 $g \cdot L^{-1}$ yeast extract, 1.7 $g \cdot L^{-1}$ YNB and 0.55 $g \cdot L^{-1}$ ammonium sulfate; and (3) VFAs-based medium supplemented with 20 $g \cdot L^{-1}$ glucose (ammonium sulfate concentration in this medium was 1.05 $g \cdot L^{-1}$). All culture media were carried out at with initial C/N mass ratio of 75.

5.2.2.2. Two-stage batch cultures

In two-stage batch cultures (TSC), *Y. lipolytica* NCYC 2904 was grown during 24 h or 48 h in glucose medium (10 $g \cdot L^{-1}$ or 20 $g \cdot L^{-1}$ glucose, 0.5 $g \cdot L^{-1}$ yeast extract, 1.7 $g \cdot L^{-1}$ YNB and ammonium sulfate to obtain an initial C/N ratio of 75) (1^{st} stage) followed by the addition of 18 $g \cdot L^{-1}$ VFAs mixture (6 $g \cdot L^{-1}$ acetate, 6 $g \cdot L^{-1}$ propionate and 6 $g \cdot L^{-1}$ butyrate) (2^{nd} stage). TSC-1 and TSC-2 experiments were carried out with 10 $g \cdot L^{-1}$ and 20 $g \cdot L^{-1}$ of initial glucose, respectively, and the pulse of VFAs was added after 24 h. In TSC-3 experiments, cells grown in 20 $g \cdot L^{-1}$ of glucose for 48 h, followed by the addition of VFAs. All experiments were conducted at 400 rpm and 1 vvm in both cultivation stages. Additionally, the effect of oxygenation conditions was also studied, varying the agitation and aeration rates in 2^{nd} stage of two-stage batch cultures: 200 rpm and 0.5 vvm (TSC-4); 600 rpm and 1.5 vvm (TSC-5). In these experiments, yeast growth in 1^{st} stage was carried out with 20 $g \cdot L^{-1}$ of glucose for 24 h at 400 rpm and 1 vvm. Table 5.1 summarizes the main experimental conditions of two-stage batch cultures carried out in the lab-scale stirred tank bioreactor.

Table 5.1- Summary of experimental conditions of two-stage batch cultures (TSC) carried out in a 5-L stirred tank bioreactor.

Experiments	1st stage	2nd stage	
		VFAs pulse	Agitation and aeration rates
TSC-1	10 g·L^{-1} glucose	24 h	400 rpm, 1 vvm
TSC-2	20 g·L^{-1} glucose	24 h	400 rpm, 1 vvm
TSC-3	20 g·L^{-1} glucose	48 h	400 rpm, 1 vvm
TSC-4	20 g·L^{-1} glucose	24 h	200 rpm, 0.5 vvm
TSC-5	20 g·L^{-1} glucose	24 h	600 rpm, 1.5 vvm

1st stage corresponds to the growth phase in glucose medium (24 h or 48 h); 2nd stage corresponds to the period after the addition of VFAs; VFAs pulse corresponds to the time at which a mixture of 18 g·L^{-1} VFAs was added; all experiments were conducted at 400 rpm and 1 vvm during the 1st stage of cultivation

5.2.3. Analytical methods

Samples were collected at appropriate intervals for the measurement of biomass, glucose and VFAs concentration, cellular lipids content, and long-chain fatty acids composition. The analysis of samples was performed as described in the chapter 3.2.6.

5.2.4. Estimation of biodiesel properties

The main properties of biodiesel (degree of unsaturation, oxidation stability, cetane number, iodine value, cloud point, cold filter plugging point, higher heating value, saponification value, pour point, kinematic viscosity and density) were estimated based on the fatty acids composition using the BiodieselAnalyzer© Ver. 2.2 (available on "http://www.brteam.ir/biodieselanalyzer") (Talebi et al. 2014).

5.2.5. Statistical analysis

One-way analysis of variance (ANOVA) was used for the statistical analysis of the data as described in chapter 3.2.8.

5.3. Results and Discussion

5.3.1. Microbial lipids production in batch cultures

The ability of *Y. lipolytica* NCYC 2904 to grow in VFAs as sole carbon and energy source was demonstrated in previous works carried out in Erlenmeyer flask experiments (Pereira et al. 2021). Herein, batch cultures were performed in a STR lab-scale bioreactor and the cellular growth and microbial lipids production were evaluated in a mixture of 18 $g \cdot L^{-1}$ VFAs, in glucose medium and in a mixture of 18 $g \cdot L^{-1}$ VFAs supplemented with glucose (Figure 5.1).

Yarrowia lipolytica NCYC 2904 was able to grow in a mixture of VFAs as sole carbon source (Figure 5.1A), but in glucose medium, a higher cellular concentration (Figure 5.1B) and biomass yield were obtained (Table 5.2). The addition to VFAs-based medium of a co-substrate (glucose), easily assimilable by the yeast, improved *Y. lipolytica* growth rate and final biomass production (Figure 5.1C). The stationary phase was reached after 48 h of cultivation in VFAs medium (coinciding with the depletion of VFAs from culture medium) and glucose medium, while in VFAs-based medium supplemented with glucose, yeast cells are still growing owing to the glucose present in the culture medium. The biomass yield on VFAs-based medium and on VFAs-based medium supplemented with glucose was similar (Table 5.2). In VFAs-batch cultures, the absence of a prolonged lag phase and the values of biomass yield obtained (0.32 $g \cdot g^{-1}$) demonstrate that *Y. lipolytica* NCYC 2904 has a good tolerance to VFAs. Similarly, Llamas et al. (2020) found that *Y. lipolytica* ACA DC 5010 was able to grow in a 15 $g \cdot L^{-1}$ VFAs mixture, attaining a biomass yield of 0.33 $g \cdot g^{-1}$. In alkaline conditions, a biomass yield of 0.167 $g \cdot g^{-1}$ was obtained for *Y. lipolytica* CICC 31596 cultivated in a mixture with 50 $g \cdot L^{-1}$ of VFAs (Gao et al. 2020). According to some authors, VFAs concentrations above 5 $g \cdot L^{-1}$ have an inhibitory effect on *Y. lipolytica* (Fontanille et al. 2012) and *Cryptococcus albidus* (Fei et al. 2011b), mainly owing to the intracellular acidification of cells, caused by undissociated form of VFAs, which induces stress on yeast metabolism and affects cell growth.

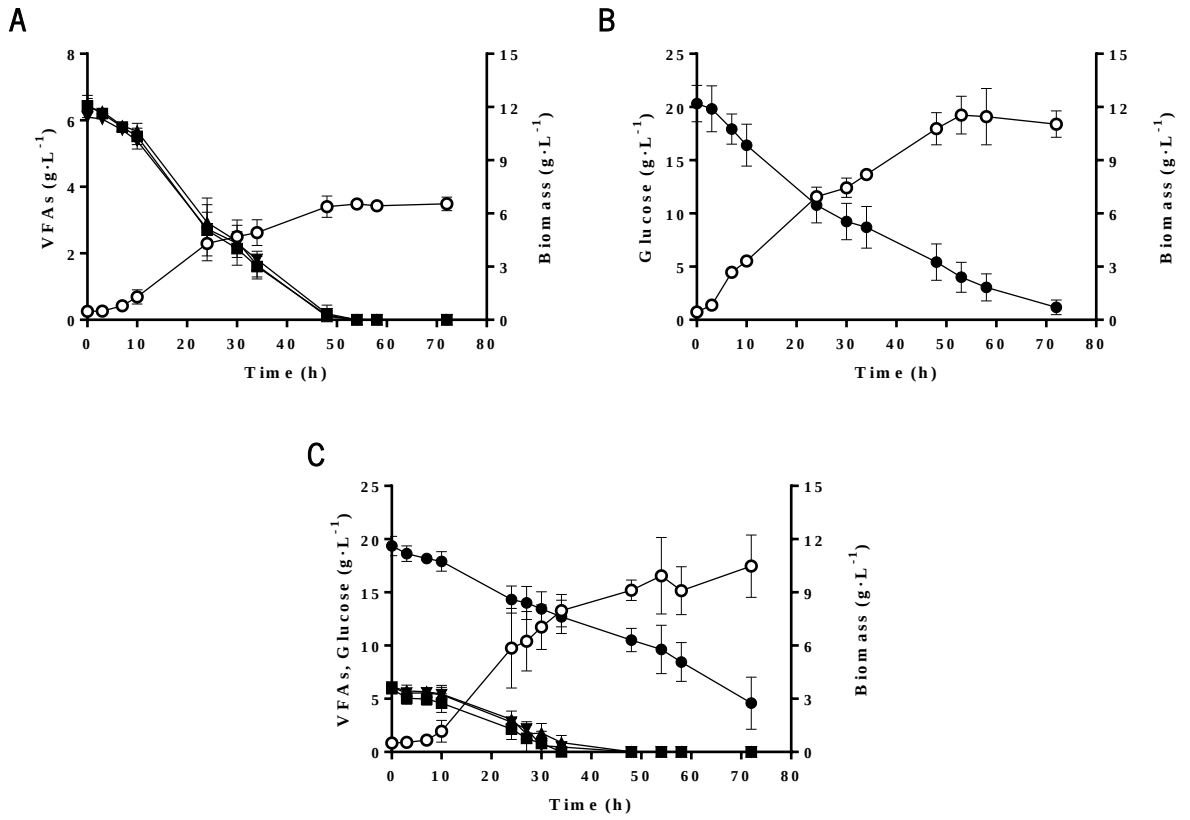

Figure 5.1 - Time course of cellular growth (○) and VFAs (■, acetate; ▲, propionate; ▼, butyrate), and glucose (●) concentration obtained in *Y. lipolytica* NCYC 2904 batch cultures carried out in a STR bioreactor with VFAs-based medium (A), glucose medium (B), and VFAs-based medium supplemented with glucose (C). The error bars represent the standard deviation of two independent replicates.

The uptake of VFAs was always associated with *Y. lipolytica* growth and all VFAs were totally consumed by yeast, even when glucose was used as co-substrate. In VFAs-based medium, all carboxylic acids were assimilated after 48 h of cultivation (Figure 5.1A), whereas in the presence of glucose a complete VFAs consumption was observed earlier, probably owing to the higher biomass concentration (Figure 5.1C). The VFAs uptake rate was not affected by the addition of a second carbon source (Table 5.2), indicating that glucose had no catabolic repression on VFAs consumption. At the end of experiments, approximately 1 $g{\cdot}L^{-1}$ and 5 $g{\cdot}L^{-1}$ of glucose remained in the glucose medium (Figure 5.1B) and in the VFAs-based medium supplemented with glucose (Figure 5.1C), respectively. In fact, a 1.7-fold decrease on glucose uptake rate was observed in presence of VFAs (Table 5.2), indicating a preference of the yeast to assimilate VFAs over glucose was observed. The total VFAs uptake rate was approximately 2-fold higher than glucose uptake rate, regardless if they were used as sole carbon source or supplemented with glucose. Moreover, 18 $g{\cdot}L^{-1}$ VFAs were completely consumed after 34 h of cultivation, whereas 20 $g{\cdot}L^{-1}$

glucose was not totally assimilated within 72 h of experiments (Figure 5.1C). In general, the metabolism of mixed substrates is summarized by sequential (e.g., diauxic) and hierarchical (yet simultaneous) utilization, based on substrate preference as a result of the evolutionary aptitude of yeast cells in their native environments (Park et al. 2019). Moreover, shorter metabolic pathways are required for VFAs metabolization compared with glucose, which is processed through complex biochemical reactions in glycolysis (Chatterjee and Mohan 2020).

Table 5.2- Values of biomass yield ($Y_{X/S}$), maximum lipids yield ($Y_{L/S}$), maximum lipids concentration, total VFAs uptake rate (R_{VFAs}) and glucose uptake rate ($R_{glucose}$) obtained in *Y. lipolytica* NCYC 2904 batch cultures. Data are average ± standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences ($p \geq 0.05$).

Carbon source	$Y_{X/S}$ ($g \cdot g^{-1}$)	$Y_{L/S}$ ($g \cdot g^{-1}$)	Lipids concentration ($g \cdot L^{-1}$)	R_{VFAs} ($g \cdot L^{-1} \cdot h^{-1}$)	$R_{glucose}$ ($g \cdot L^{-1} \cdot h^{-1}$)
VFAs	0.32 ± 0.03 [a]	0.032 ± 0.006 [a]	0.33 ± 0.06 [a]	0.42± 0.01 [a]	-
Glucose	0.55 ± 0.01 [b]	0.09 ± 0.01 [b]	1.7 ± 0.04 [b]	-	0.32 ± 0.02 [a]
VFAs + Glucose*	0.32 ± 0.05 [a]	0.046 ± 0.007 [a]	1.6 ± 0.3 [b]	0.43 ± 0.02 [a]	0.19 ± 0.03 [b]

$Y_{X/S}$ was calculated at the end of cellular growth (72 h); $Y_{L/S}$ was calculated at 24 h of cultivation for VFAs-based medium and at 72 h for glucose medium and VFAs-based medium supplemented with 20 $g \cdot L^{-1}$ glucose; * $Y_{X/S}$ and $Y_{L/S}$ were calculated as cell mass and lipid mass, respectively, per mass of total substrates (VFAs and glucose) consumed.

Unlike to the results reported by Gao et al. (2017) and Pereira et al. (2021), all VFAs in the mixture (acetate, propionate and butyrate) were consumed at similar uptake rates. It is worth to highlight that total VFAs uptake rates obtained in this work (0.42 $g \cdot L^{-1} \cdot h^{-1}$ and 0.43 $g \cdot L^{-1} \cdot h^{-1}$) are higher than others described in the literature for *Y. lipolytica* batch cultures. In *Y. lipolytica* CICC 31596 cultures, a mixture of 2.5 $g \cdot L^{-1}$ of VFAs was consumed within an uptake rate of 0.04 $g \cdot L^{-1} \cdot h^{-1}$ (Gao et al. 2017). The total VFAs uptake rate of *Y. lipolytica* W29 ranged between 0.21 $g \cdot L^{-1} \cdot h^{-1}$ (Erlenmeyer flasks) and 0.28 $g \cdot L^{-1} \cdot h^{-1}$ (bioreactor experiments) in batch cultures with a mixture of 6 $g \cdot L^{-1}$ VFAs. Moreover, when the concentration of total VFAs raised to 18 $g \cdot L^{-1}$ in Erlenmeyer flask experiments, the VFAs uptake rate slightly decreased to 0.19 $g \cdot L^{-1} \cdot h^{-1}$ (Pereira et al. 2021).

The accumulation of microbial lipids by *Y. lipolytica* from hydrophilic substrates (e.g. glucose and acetate) involves the formation of fatty acid precursors, such as acetyl-CoA and malonyl-CoA, and their integration

into *de novo* lipid biosynthesis pathway (Papanikolaou and Aggelis 2011b). This metabolic route is induced by the exhaustion from the culture medium or limitation of a primary nutrient, usually nitrogen. When nitrogen becomes unavailable, cell proliferation slows down, since it is an essential nutrient for protein and nucleic acid synthesis. However, the cell continues assimilating the carbon sources from medium, which are now channeled to lipid synthesis (Papanikolaou and Aggelis 2011b; Karamerou and Webb 2019). On the other hand, it has been reported that microbial lipid accumulation from propionate and butyrate occurred via *ex novo* lipid biosynthesis pathway (Morales-Palomo et al. 2022). E*x novo* lipid biosynthesis pathway is independent of nitrogen limitation in the culture medium (Papanikolaou and Aggelis 2011b).

In this work, the accumulation of microbial lipids by *Y. lipolytica* NCYC 2904 showed 3 different patterns, depending on substrate used (Figure 5.2): (1) in VFAs-based medium, lipids content decreased through cultivation time and the amount of lipids at 72 h was half that obtained at 24 h; (2) in glucose medium, lipids production increased until the end, and a 5-fold improvement in lipids content was obtained after 72 h comparatively to 24 h; and (3) in VFAs-based medium supplemented with glucose, no statistical differences were observed in intracellular lipids through time. In VFAs-based medium, the maximum lipids accumulation occurred during the exponential growth phase and a mobilization of synthesized lipids for cellular metabolic activities was observed in stationary phase, since substrate was completely consumed after 48 h of cultivation (Figure 5.1A and 5.2). By contrast, in media with glucose, sugar was not completely consumed until the end of cultivation (Figure 5.1B), which may have contributed to prevent lipid turnover. Moreover, the maximum lipids accumulation occurred in stationary growth phase, indicating that the glucose consumed in this period was channeled toward lipids production, instead of being used for biomass synthesis.

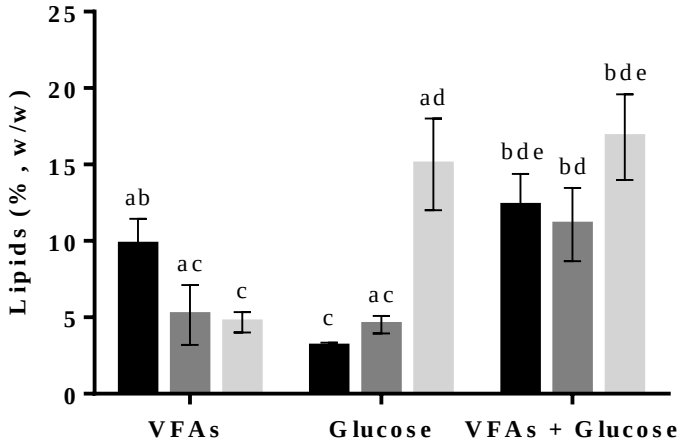

Figure 5.2 - Microbial lipids content of *Y. lipolytica* NCYC 2904 cells obtained in batch cultures growing in VFAs-based medium, glucose medium and VFAs-based medium supplemented with glucose after 24 h (black bars), 48 h (grey bars) and 72 h (light grey bars) of cultivation. The error bars represent the standard deviation of two independent replicates. Bars with the same letter do not present statistically significant differences ($p \geq 0.05$).

The addition of a co-substrate to VFAs-based medium enabled the utilization of carbon sources for simultaneous production of intracellular lipids and biomass. The carbon-to-bioproducts conversion may vary in mixed substrates, since each substrate has unique efficiencies for carbon, energy and cofactors generation. Furthermore, mixtures of substrates with distinct entry points to metabolism provide the required components in fewer enzymatic steps, relieving protein burdens and enhancing metabolic pathways efficiency (Park et al. 2019). In fact, at each time, the lipids content obtained in VFAs-based medium supplemented with glucose was approximately the sum of the amounts attained with individual substrates (Figure 5.2). Park et al. (2019) observed an improvement of *Y. lipolytica* growth and lipids production by using a fed-batch co-feeding strategy. The synergistic effect of glucose (secondary substrate) and acetate (primary substrate) raised product synthesis by enhancing NADPH generation. Lipogenesis in *Y. lipolytica* requires the supply of ATP, acetyl-CoA and large amounts of the reducing cofactor NADPH, which primary source is the oxidative pentose phosphate pathway (PPP). Although both acetate and glucose, used as single substrates, provide all these building blocks for lipids synthesis, glucose can flow more directly to PPP and generates NADPH more efficiently. On the other hand, acetate supports the generation of ATP and acetyl-CoA through the tricarboxylic acid cycle (TCA), but not NADPH formation, which requires several enzymatic steps and ATP (Qiao et al. 2015; Liu et al. 2016).

The maximum lipids yield was not significantly improved by the addition of a co-substrate (Table 5.2), suggesting that the additional substrate was channeled towards the production of other metabolites, or even for cellular growth, instead of microbial lipids. The intermediate acetyl-CoA produced from VFAs can incorporate the glyoxylate shunt or be transported into the mitochondria and enter in the TCA cycle. Glucose is metabolized through glycolysis and TCA cycle, producing other compounds (Fickers et al. 2005; Papanikolaou et al. 2009). In fact, the maximum lipids concentration was improved by the addition of glucose (Table 5.2), owing to the higher biomass concentration reached in this condition (Figure 5.1C). The enhancement of lipids production by yeast cells were also obtained in cultivations on VFAs (Kolouchová et al. 2015; Pereira et al. 2021), frying vegetable oils (Bialy et al. 2011) and n-alkanes (Matatkova et al. 2017), after addition of glucose as co-substrate. The maximum value of lipids production in batch cultures (1.6 $g \cdot L^{-1}$) is similar to others found in the literature for *Y. lipolytica* cells growing in VFAs. In a mixture of 6 $g \cdot L^{-1}$ VFAs supplemented with glycerol or glucose, *Y. lipolytica* W29 cells produced, respectively, 0.82 $g \cdot L^{-1}$ and 1.4 $g \cdot L^{-1}$ of lipids (Pereira et al. 2021). The production of lipids by *Y. lipolytica* CICC 31596 was dependent on type and concentration of VFAs, ranging from 0.55 $g \cdot L^{-1}$ (butyric acid 2.5 $g \cdot L^{-1}$) to 1.17 (acetic acid 10 $g \cdot L^{-1}$) (Gao et al. 2017). The use of 50 $g \cdot L^{-1}$ of mixed VFAs, at pH 6, led to an improvement of lipids concentration up to 3.16 $g \cdot L^{-1}$ (Gao et al 2020).

Microbial lipids produced in VFAs-based medium were predominantly composed of oleic and linoleic acids, whereas in media with glucose (used individually or mixed with VFAs), oleic acid was by far the main fatty acid (Table 5.3). Microbial lipids produced by *Y. lipolytica* NCYC 2904 in batch cultures were mostly composed by unsaturated fatty acids (> 82 %), showing the high similarity of these lipids with vegetable oils, traditionally used as raw material for biodiesel production. The addition of glucose to VFAs led to an increase of oleic, palmitic, and palmitoleic acid content and a considerable reduction of linoleic acid. Moreover, a slight decrease of the unsaturated fraction was observed in media supplemented with glucose comparatively to VFAs-based medium. According to Silverman et al. (2016), *Y. lipolytica* growing in glucose produces a lipid profile highest in oleic acid and much lower in linoleic acid, regardless if yeast strains are overexpressing lipogenesis-related genes or not. Furthermore, it is noteworthy the synthesis of odd-chain fatty acids, namely heptadecenoic and margaric acids in VFAs-based medium and VFAs-based medium supplemented with glucose. Odd-chain fatty acids have gained considerable attention due to a wide range of applications in nutrition and pharmaceutical industries, as well as in chemical industries, including biodiesel production improving its fuel properties (Park et al. 2018). The results obtained in batch cultures indicate that VFAs can be used as raw material by *Y. lipolytica* for biosynthesis of specialty lipids with high added-value.

Table 5.3 - Fatty acids composition of microbial lipids produced by *Y. lipolytica* NCYC 2904 in batch cultures. Data are average ± standard deviation for two independent replicates. Values followed by the same letter in each column do not present statistically significant differences ($p \geq 0.05$).

Carbon Source	Relative fatty acid content (%)						
	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2
VFAs	6.9 ± 0.3 [a]	3.5 ± 0.1 [a]	0.9 ± 0.1 [a]	12.3 ± 0.2 [a]	3.6 ± 0.9 [a]	42.8 ± 0.4 [a]	30 ± 1 [a]
Glucose	12.3 ± 0.1 [b]	12.9 ± 0.2 [b]	-	-	4.3 ± 0.6 [a]	57.9 ± 0.7 [b]	12.6 ± 0.5 [b]
VFAs + Glucose	11.4 ± 0.3 [c]	7.6 ± 0.2 [c]	2.3 ± 0.4 [b]	8.1 ± 0.1 [b]	4.0 ± 0.0 [a]	53.1 ± 0.4 [c]	13.5 ± 0.8 [b]

5.3.2. Microbial lipids production in two-stage batch cultures

A two-stage batch culture strategy (growth in glucose – 1st stage – followed by the addition of 18 $g \cdot L^{-1}$ VFAs – 2nd stage) was studied as an attempt to enhance microbial lipids production.

Firstly, *Y. lipolytica* NCYC 2904 cells grew in 10 $g \cdot L^{-1}$ (TSC-1) or 20 $g \cdot L^{-1}$ glucose during 24 h (TSC-2) or 48 h (TSC-3), followed by the addition of 18 $g \cdot L^{-1}$ VFAs. After the addition of VFAs (2nd stage), no significant cellular growth was observed and the total biomass yield was statistical equal in all experiments (Figure 5.3, Table 5.4). In 2nd stage, no extra nitrogen source was added, which led to nitrogen-limitation, stopping the microbial cells growth (Rane et al. 2021). However, VFAs were completely consumed, suggesting that this carbon source was used for other purposes (e.g., cellular maintenance, synthesis of lipids or other metabolites) than biomass production. Regardless of the time at which the pulse of VFAs was added, a metabolic shift in *Y. lipolytica* cells was observed in 2nd stage, since glucose assimilation slowed down and yeast cells readily started consuming VFAs (Figure 5.3). In fact, glucose uptake rates in 2nd stage (0.02 $g \cdot L^{-1} \cdot h^{-1}$ - 0.05 $g \cdot L^{-1} \cdot h^{-1}$) were considerably lower than those obtained in the 1st stage (0.31 $g \cdot L^{-1} \cdot h^{-1}$ - 0.43 $g \cdot L^{-1} \cdot h^{-1}$). The total VFAs uptake rate was not affected by the amount of glucose in the culture medium at the moment of the pulse (Table 5.4). In all conditions, acetate was completely consumed 34 h after the addition of VFAs, whereas propionate took 72 h to be totally assimilated and a residual amount of butyrate still remained in media at the end of cultivation (Figure 5.3).

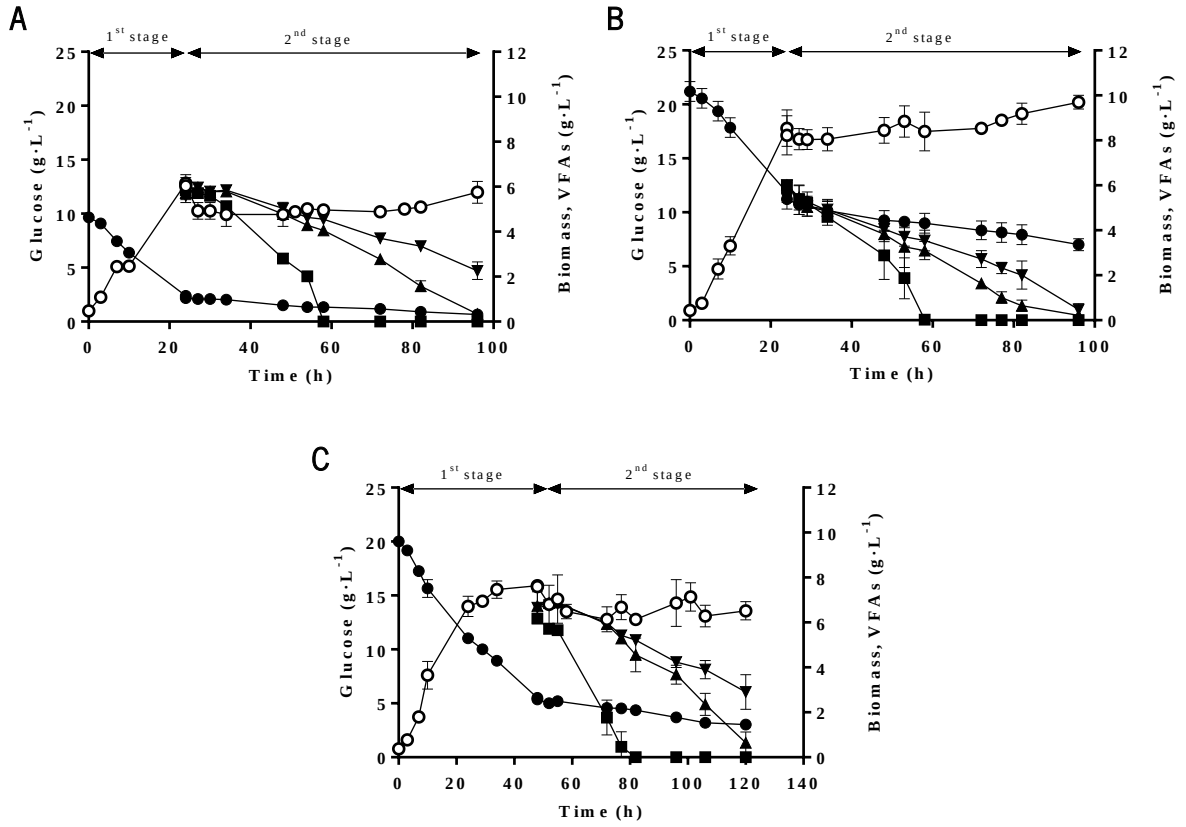

Figure 5.3 - Time course of cellular growth (○) and VFAs (■, acetate; ▲, propionate; ▼, butyrate) and glucose (●) concentration obtained in two-stage batch cultures of *Y. lipolytica* NCYC 2904: TSC-1 (A), TSC-2 (B) and TSC-3 (C). The error bars represent the standard deviation of two independent replicates.

Regardless of culture strategy, a considerable improvement of lipids content (Figure 5.4) and lipids concentration (Table 5.4) were attained in two-stage batch cultures, comparatively to those obtained in batch experiments. The maximum lipids content in TSC-2 (37 %) was considerably higher than those obtained in batch experiments carried out with VFAs (10 %) and VFAs supplemented with glucose (17 %). A two-stage batch culture was an effective approach to boost lipids synthesis by *Y. lipolytica* NCYC 2904 from VFAs. After the addition of VFAs, all carbon sources assimilated were channeled towards cell maintenance and lipids synthesis, since cell proliferation decreased, yeast cells were in stationary growth phase (Figure 5.3) and the amount of lipids increased through cultivation time (Figure 5.4). Approximately 1.5-fold improvement on lipids content was attained after 72 h of the VFAs addition, comparatively to that obtained after 24 h, both in TSC-2 and TSC-3. By contrast, in TSC-1 no significant differences were observed. Other *Y. lipolytica* strains shown similar behavior, being the production of lipid-free biomass the main process during cell proliferation phase in glucose medium, whereas lipids synthesis occurred

during the 2nd stage, after the addition of VFAs (Fontanille et al. 2012; Pereira et al. 2021). It is worth to notice that the up-scaling of two-stage batch culture from Erlenmeyer flask (Pereira et al. 2021) to lab-scale bioreactor (TSC-2, this study) led to a 1.5-fold enhancement on lipids content. This result is of upmost importance since it shows the feasibility of industrial production of microbial lipids by *Y. lipolytica* from VFAs in large scale bioreactors.

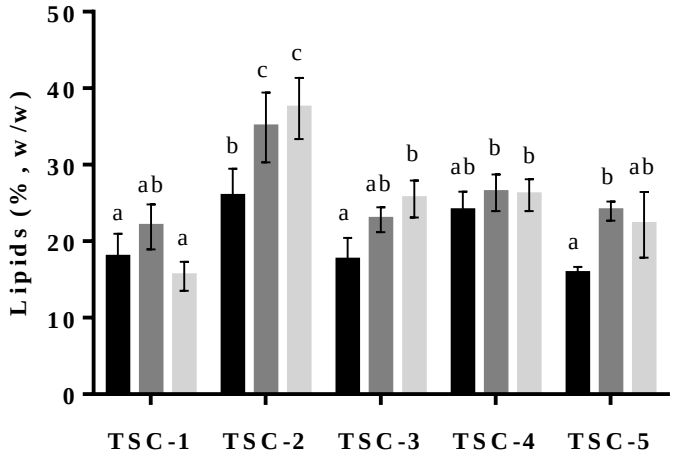

Figure 5.4 - Microbial lipids content of *Y. lipolytica* NCYC 2904 cells obtained in two-stage batch cultures after 24 h (black bars), 48 h (dark grey bars) and 72 h (light grey bars) of the addition of 18 g·L^{-1} VFAs. The error bars represent the standard deviation of two independent replicates. Bars with the same letter do not present statistically significant differences ($p \geq 0.05$).

The lipids content was significantly higher in TSC-2 (37 %) than in TSC-1 (22 %) and TSC-3 (26 %). Moreover, the maximum lipids concentration was also attained in TSC-2 (3.5 g·L^{-1}) comparatively to TSC-1 (1.1 g·L^{-1}) and TSC-3 (1.7 g·L^{-1}). The amount of glucose in culture medium during the 2nd stage of cultivation had consequences on lipid production since the lipid biosynthesis by the *de novo* pathway (as occurs from glucose) is affected by the concentration of carbon and nitrogen sources. Moreover, it is noted the preference of *Y. lipolytica* for acetate (Figure 5.3), which is uptaken via the *de novo* pathway (Morales-Palomo et al., 2022). In *Y. lipolytica*, *de novo* lipid synthesis is a secondary anabolic biochemical process that requires an excess of carbon source, depletion of extracellular nitrogen, and relatively high C/N ratio to trigger the onset of lipogenesis. At the moment of VFAs addition and until the end of cultivation, a higher amount of glucose remained in the TSC-2 medium than in TSC-1 and TSC-3 media (Figure 5.3), resulting in a higher C/N ratio and favoring lipid production.

Yarrowia lipolytica is a strictly aerobic yeast and its lifecycle growth, substrates consumption and metabolites production are greatly affected by the amount of oxygen available in the culture medium. However, oxygen influence on lipids biosynthesis is still not consensual and, so far, no information concerning its effect on lipids production by *Y. lipolytica* strains from VFAs in two-stage batch cultures is available in the literature. Whereas some studies indicate that highly aerated cultures favor the lipid synthesis (Tai and Stephanopoulos 2013; Bellou et al. 2014; Rakicka et al. 2015), other authors have reported that great lipids production is achieved under low aeration conditions (Magdouli et al. 2018; Lopes et al. 2018; Lopes et al. 2019). Therefore, the effect of oxygen availability on lipids synthesis by *Y. lipolytica* NCYC 2904 in two-stage batch cultures was evaluated, testing different combinations of agitation and aeration rates in growth (1st stage) and lipogenic (2nd stage) phases.

The decrease of oxygenation conditions in 2nd stage (TSC-4) had a clear negative effect on VFAs consumption (Figure 5.5A). A 4-fold decrease in VFAs consumption rate was obtained comparatively with TSC-2 experiments, in which the agitation and aeration rates were the same in both stages of cultivation (Table 5.4). Though yeast cells have consumed a low amount of glucose after the addition of VFAs, as occurred in all two-stage batch cultures, none of the VFAs were completely consumed and approximately 13 $g \cdot L^{-1}$ VFAs remained in medium in TSC-4 experiments. Additionally, no cell proliferation was observed in the 2nd stage and cells lasting in stationary growth phase until the end of cultivation. The decrease of agitation and aeration rates after the VFAs addition had a strong impact on dissolved oxygen concentration, which dropped to zero and remained in this value until the end of experiment (Figure 5.5C). The oxygen limitation observed in some cultures (e.g. TSC-4) was probably the main reason for low VFAs consumption and the absence of any cellular growth, since low oxygen concentrations decrease the activity of some enzymes involved in the metabolic pathways of the strictly aerobic yeast *Y. lipolytica* (Kamzolova et al. 2003).

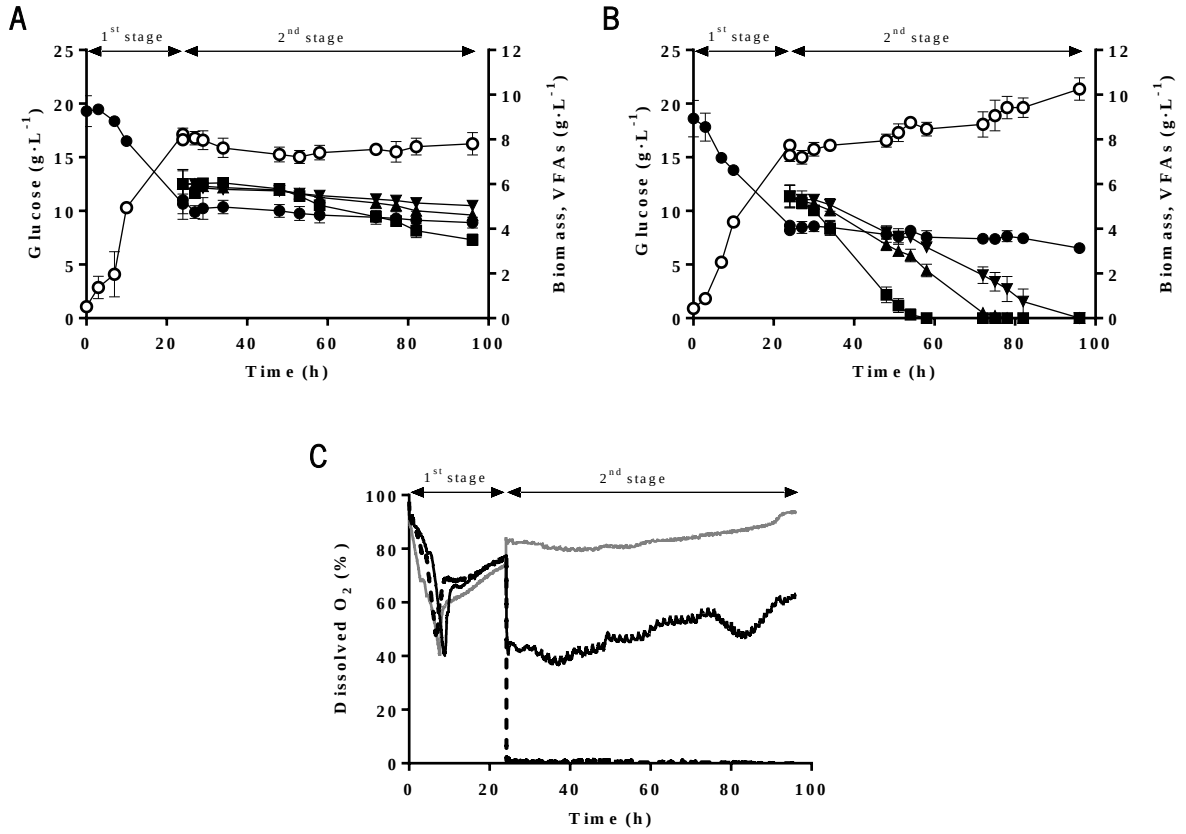

Figure 5.5 - Time course of cellular growth (○), VFAs (■, acetate; ▲, propionate; ▼, butyrate) and glucose (●) concentration obtained in two-stage cultures of *Y. lipolytica* NCYC 2904: TSC-4 (a) and TSC-5 (b); dissolved oxygen concentration (% of saturation) (c) obtained in: TSC-1 (black line), TSC-4 (black dashed line) and TSC-5 (grey line). The error bars represent the standard deviation of two independent replicates.

By contrast, raising agitation and aeration rates in 2nd stage of batch culture (TSC-5) led to a considerable increase of VFAs consumption rate (Table 5.4) and to an earlier complete assimilation of VFAs. Acetate, propionate and butyrate were completely consumed, respectively, 30 h, 48 h and 58 h after the VFAs addition (Figure 5.5B). In these experiments, the dissolved oxygen concentration in the 2nd stage never dropped to zero and stabilized between 80 % and 95 % of saturation (Figure 5.5C). The increase of agitation and aeration allowed to maintain the dissolved oxygen at non-limiting conditions (e.g., TSC-5 experiment), enhancing VFAs consumption and enabling a higher yeast growth in this stage (almost 3 g·L-1). VFAs consumed by yeast in this condition were more channeled for cell proliferation than for lipid synthesis, since no considerable increase on lipids content was observed through cultivation time (Figure 5.4).

Table 5.4 - Values of total biomass yield ($Y_{X/S}$), maximum lipids yield ($Y_{L/S}$), maximum lipids concentration and total VFAs uptake rate (R_{VFAs}) obtained in *Y. lipolytica* NCYC 2904 two-stage batch cultures. Data are average ± standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences ($p \geq 0.05$).

Two-stage batch cultures	$Y_{X/S}$ ($g \cdot g^{-1}$)	$Y_{L/S}$ ($g \cdot g^{-1}$)	Lipids concentration ($g \cdot L^{-1}$)	R_{VFAs}* ($g \cdot L^{-1} \cdot h^{-1}$)
TSC-1	0.22 ± 0.01 [a]	0.042 ± 0.001 [a]	1.1 ± 0.1 [a]	0.22 ± 0.02 [a]
TSC-2	0.29 ± 0.02 [a]	0.10 ± 0.01 [b]	3.5 ± 0.4 [b]	0.24 ± 0.01 [a]
TSC-3	0.1865 ± 0.004 [a]	0.036 ± 0.006 [a]	1.7 ± 0.2 [c]	0.24 ± 0.02 [a]
TSC-4	0.6 ± 0.1 [b]	0.11 ± 0.01 [b]	2.0 ± 0.2 [c]	0.064 ± 0.007 [b]
TSC-5	0.29 ± 0.05 [a]	0.07 ± 0.01 [c]	2.0 ± 0.2 [c]	0.27 ± 0.01 [a]

*Values correspond to 2nd stage of cultivation; $Y_{X/S}$ and $Y_{L/S}$ were calculated as cell mass and lipid mass, respectively, per mass of total substrates (VFAs and glucose) consumed.

Though some studies indicate that great amounts of lipids are synthesized in lower oxygenation conditions (Lopes et al. 2018; Lopes et al. 2019), the decrease of agitation and aeration during lipogenic phase had a negative effect on lipids synthesis by *Y. lipolytica* NCYC 2904. In these conditions, dissolved oxygen concentration in the medium remained nearly to zero in all lipogenic phase (Figure 5.5C), which was clearly insufficient for metabolic reactions of cellular growth and lipids synthesis. Moreover, oxygen is the final electron acceptor in yeast respiratory chain, in which the intermediate NADPH, that are used in lipids synthesis, is produced (Silverman et al. 2016; Zhang et al. 2019b). Magdouli et al. (2018) reported that the highest microbial lipids production by *Y. lipolytica* SM7 from crude glycerol was obtained under a dissolved oxygen concentration of 30 % of saturation. In fact, in the present study, lipids synthesis was enhanced in the condition where dissolved oxygen stabilized between 40 % and 50 % of saturation (TSC-2) during lipogenic phase. At these non-limiting conditions, assimilated VFAs were mainly directed to lipids metabolism pathways than cellular growth. By contrast, in highly aerated conditions (dissolved oxygen concentration above 80 % of saturation) during lipogenic phase (TSC-5), lipid synthesis was negatively affected. According to Bellou et al. (2014), lipids synthesis by *Y. lipolytica* is favored by high dissolved oxygen concentration as a result of the upregulation of enzymes involved in the lipids metabolism, such

as ATP-citrate lyase and malic enzyme. In the 2nd stage of cultivation, a significant cellular growth was observed, which means that, in this condition, VFAs consumed were more channeled towards cellular growth than for lipids synthesis comparatively to the other experiments. This result is in accordance with previous work of Papanikolaou et al. (2007), in which *Y. lipolytica* metabolism was drove toward lipid-free biomass synthesis instead of lipids production in the experiments carried out with high oxygen concentration (60 % - 70 % of saturation) during lipogenesis.

It was demonstrated that using the same agitation and aeration rates during growth and lipogenic phases (allow to maintain the dissolved oxygen concentration between 40 % and 50 % of saturation) enhanced lipids content, lipids yield and lipids concentration. This is an important advantage for industrial production of microbial lipids, since the costs imputed to the mechanical agitation and aeration will not increase during lipogenic phase. Moreover, it must be stressed out that the maximum lipids content obtained in this work (37 %, w/w) is one of the highest found in literature for a *Y. lipolytica* wild strain growing in VFAs-based medium.

The fatty acids composition of lipids accumulated by *Y. lipolytica* NCYC 2904 cells in all two-stage batch cultures was similar to that obtained in batch cultures. Oleic acid (53 % ± 3 %) was the predominant fatty acid, followed by linoleic (16 % ± 3 %), palmitic (9 % ± 2 %), palmitoleic (8 % ± 2 %) and stearic (4.6 % ± 0.8 %) acids. Once again, the synthesis of odd-chain fatty acids – heptadecenoic (9 % ± 4 %) and margaric (1.9 % ± 0.3 %) acids – was observed due to the presence of propionate in the lipogenic phase. Furthermore, the unsaturated fraction (> 84 %) was not affected by glucose concentration and oxygenation conditions. The fatty acid composition of microbial lipids synthesized by *Y. lipolytica* NCYC 2904 from VFAs, similar to vegetable oils, suggests that they can be used as raw material for biodiesel production.

5.3.3. Estimation of biodiesel properties obtained from *Y. lipolytica* lipids

The quality of biodiesel relies on several parameters, such as density, viscosity, heating value, cetane number, oxidation stability, cold flow properties, cold filter plugging point, among others. These properties are required to assess the potential of biodiesel to replace conventional diesel fuel. The fatty acids profile of oils used in the transesterification reactions, namely unsaturation degree, number and position of double bonds and fatty acids chain length, strongly affects the biodiesel properties and are considered crucial to produce high quality biodiesel. The amount of unsaturated fatty acids, for example, define the biodiesel cold flow plugging properties, whereas the presence of high saturated fatty acids avoids the auto-oxidation of biodiesel and increases its shelf-life (Patel et al. 2017). The fatty acids composition of microbial lipids produced by *Y. lipolytica* NCYC 2904 in TSC-2 (condition where higher amount of lipids

was obtained) was used to predict properties of biodiesel. Table 5.5 shows the main parameters used to assess the biodiesel quality, estimated by BiodieselAnalyzer© software, and compared with international biodiesel standards EN 14214 and ASTM D6751.

Table 5.5 - Physiochemical properties of biodiesel obtained from *Y. lipolytica* NCYC 2904 lipids estimated by BiodieselAnalyzer© software.

Properties	Estimated results	US biodiesel standard ASTM D6751	EU biodiesel standard EN 14214
Density ($kg \cdot m^{-3}$)	874	NR	860 – 900
Kinematic viscosity ($mm^2 \cdot s^{-1}$)	3.93	1.9 – 6.0	3.5 – 5.0
Higher heating value (MJ/kg)	39.47	NR	NR
Cetane number	54.81	51 min	47 min
Iodine value	82.05	NR	120 max
Cloud point (°C)	- 0.84	-12 to -3	NR
Oxidation Stability (h)	9.83	3 h min	6 h min
Saponification value ($mg \cdot g^{-1}$)	202.39	310 max	NR
Cold filter plugging point (°C)	- 6.61	NR*	NR*
Pour Point (°C)	- 7.73	-15 to -16	NR
Degree of unsaturation	90.30	NR	NR

NR – not reported; NR*– CFPP limits depend on geography and time of the year; min – minimum; max – maximum; h – hours

High heating value (HHV) of the fuel is one of the most important property, since it defines the heating energy released during the combustion and thereby the efficiency of biodiesel (Fassinouet al., 2010). The HHV estimated in this work (39.47 MJ/Kg) is consistent with others encountered in literature for biodiesel obtained from waste cooking oil (40.11 MJ/kg), soybean oil (39.77 MJ/kg) and lipids of *Y. lipolytica* grown in non-detoxified liquid wheat straw hydrolysate (41.71 MJ/kg), in detoxified liquid wheat straw hydrolysate (41.63 MJ/kg) and in glucose (42.47 MJ/kg) (Fassinouet al., 2010; Nambou et al., 2014). Another relevant parameter for biodiesel quality is the cold filter plugging point (CFPP), as it determines the lowest temperature at which fuels easily flows through a standard filter in a specific time. The crystallization or gelling that may occur in biodiesel at low temperatures have severely negative effects in the engine performance as they may clog the fuel line and filters (Patel et al. 2017). The CFPP estimated herein (– 6.61 °C) is similar to those reported by Yaşar (2020) using several vegetable oils (between – 13 °C and – 4 °C), higher than those determined by Patel et al. (2017) using *Y. lipolytica* lipids (– 13.16

°C and – 11 °C) and lower than the value obtained by Brar et al. (2017) using lipids of *Trichosporon sp.* grown on glucose (18.5 °C), glycerol (0.87 °C) and xylose rich acid hydrolysate of sugarcane bagasse (11.6 °C). It is worth to notice that, as lower is the CFPP value, as best are the cold flow properties of the biodiesel.

Cetane number (CN), an indicator of ignition characteristics of fuel, is related with the time interval between the start of ignition and the beginning of combustion and affects several parameters of engine performance, such as stability, noise and CO emissions. A CN higher than 65 causes an instant ignition in a short distance to the injector nozzle, without a proper mixing of air, resulting in an excessive heating of the injector and a reduction of biodiesel efficiency. Low CN of biodiesel is inconvenient, as well, since it affects the engine starting in cold environments and subsequent generation of noise, white smoke and hydrocarbons emissions (Patel et al. 2017; Yaşar 2020). The estimated CN for biodiesel obtained from microbial lipids of *Y. lipolytica* NCYC 2904 (54.81) meets this criterion and is similar to CN of biodiesel produced from canola and rapeseed oils (Yaşar 2020). Other estimated properties, such as density, kinematic viscosity, iodine value and oxidation stability meet the criteria set up by international biodiesel standards EN 14214 and ASTM D6751 and are similar to those of biodiesel produced from vegetable oils (Patel et al. 2017; Yaşar 2020). Therefore, microbial lipids produced by *Y. lipolytica* NCYC 2904 from VFAs are highly suitable for high quality biodiesel production.

5.4. Conclusions

Yarrowia lipolytica NCYC 2904 was able to grow and produce significant amounts of microbial lipids from high-content VFAs-based media, particularly in two-stage batch cultures. C/N ratio and dissolved oxygen concentration in lipogenic phase proved to be important factors affecting lipids production in these cultures. The dissolved oxygen at a non-limiting concentration in the 2nd phase (40 % - 50 % of saturation) enhanced lipids biosynthesis up to 37 % (w/w), one of the highest values found in the literature for a *Y. lipolytica* wild strain in VFAs-based media. Microbial lipids of *Y. lipolytica* NCYC 2904 were highly unsaturated and mainly composed by oleic acid. This profile, similar to vegetable oils, make these lipids a suitable raw material for biodiesel industry. In fact, the estimated properties of biodiesel obtained from these intracellular lipids, proved their potential as feedstock for the production of high-quality biofuel. The bioprocess herein proposed is a step forward in food wastes valorization, by using the intermediates VFAs (obtained by anaerobic fermentation), as a substrate for microbial lipids production. The exploitation of *Y. lipolytica* NCYC 2904 as a biorefinery platform to obtain high-value lipids for biodiesel industry from

renewable and low-cost raw materials represents an environmentally friendly strategy, fulfilling the circular economy guidelines.

CHAPTER 6

Integrated bioprocess of microbial lipids production in *Yarrowia lipolytica* using food-waste derived volatile fatty acids

This chapter is based on the following original research article:

Pereira, A. S., Lopes, M, Duarte, M.S., Alves, M.M., Belo, I. (2023) Integrated bioprocess of microbial lipids production in *Yarrowia lipolytica* using food-waste derived volatile fatty acids. Renewable Energy, 202: 1470-1478. https://doi.org/10.1016/j.renene.2022.12.012

6.1. Introduction

The increasing growth of the human population and the global economy has resulted in the production of large quantities of food waste (FW). According to the Food and Agriculture Organization of the United Nations (FAO), approximately 931 million tons of food are wasted globally (17 % of total global food production) (UNEP 2021). Traditionally, landfilling and incineration are the most common food waste management approaches, but are also the least sustainable ones. The improper management of food waste triggers environmental problems, such as greenhouse gas emissions, deterioration of water quality, and contamination of the land area (Usmani et al. 2021). The environment-friendly technology anaerobic digestion (AD) has been widely used to convert food wastes into bioenergy (biogas) and valuable biochemicals, such as volatile fatty acids (VFAs) (Duarte et al. 2021; Usmani et al. 2021). This process consists of four consecutive stages - hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Volatile fatty acids are important intermediates produced mainly during the acidogenic stage and their production has attracted great interest since they are considered important building blocks for chemical industries and can also be used as the carbon source for oleaginous yeasts (Llamas et al., 2020b; Patel et al., 2021; Pereira et al., 2021). In general, the concentration of VFAs generated by anaerobic fermentation of food waste ranges from 4 $g \cdot L^{-1}$ to 40 $g \cdot L^{-1}$, and composition depends on substrate type and fermentation conditions. Acetic, propionic, and *n*-butyric acids are generally the three major carboxylic acids representing more than 80 % of the total produced VFAs. Among the remaining VFAs produced are iso-butyric, *n*-valeric, iso-valeric, *n*-caproic, and iso-caproic acids (Gameiro et al. 2016; Gao et al. 2017; Moretto et al. 2019a; Gao et al. 2020; Duarte et al. 2021).

The oleaginous yeast *Yarrowia lipolytica* has proved to be an attractive platform for microbial lipids production from a wide range of substrates, including non-refined ones (Lopes et al. 2022). Lipids of *Y. lipolytica* are predominantly composed of C16-C18 fatty acids - similar to vegetable oils - making these lipids a suitable feedstock for the biodiesel industry (Pereira et al. 2021; Pereira et al. 2022b). However, the utilization of microbial lipids for biodiesel production is still hindered by the high costs of pure substrates used in yeast cultivation (Koutinas et al. 2014). The utilization of low-cost feedstocks is currently presented as an attractive option to reduce global production costs. Given the ability of *Y. lipolytica* to grow and accumulate lipids using carbon sources derived from organic wastes (Lopes et al. 2022), this study proposes an alternative approach for the economical and sustainable production of microbial lipids by *Y. lipolytica* from FW-derived VFAs. Several works on lipids production have been developed with pure VFAs, but few authors studied the use of VFAs produced during the acidogenesis of organic wastes as a substrate for *Y. lipolytica* growth and lipids accumulation. Thus, the current study

evaluated the feasibility of using VFAs produced during the acidogenesis of food waste, wastewater derived from the biodiesel industry, and brine wastewater from a fish-canning industry, as the carbon source for *Y. lipolytica* (W29 and NCYC 2904) growth and microbial lipids production. Batch cultures of *Y. lipolytica* in flask experiments were performed in FW-derived VFAs and with crude glycerol as co-substrate in order to evaluate the potential of this low-cost feedstock for practical application. Additionally, the performance of *Y. lipolytica* NCYC 2904 batch cultures in FW-derived VFAs medium was evaluated in a laboratory-scale bioreactor (stirred tank bioreactor). A two-stage batch culture using FW-derived VFAs medium to cell proliferation (growth phase) and crude glycerol to lipid synthesis (lipogenic phase) was carried out, for the first time, as an attempt to boost biomass and lipid production by *Y. lipolytica* NCYC 2904.

6.2. Materials and Methods

6.2.1. Yeast strains and inoculum preparation

Yarrowia lipolytica W29 and *Y. lipolytica* NCYC 2904 cells were preserved in cryo-stocks at - 80 °C and pre-inoculum was prepared as described in chapter 3.2.1.

6.2.3. Anaerobic fermentation

The production of VFAs was carried out in 2 Expanded Granular Sludge Bed (EGSB) reactors of 2 L with external recirculation and coupled to a settler. Anaerobic granular sludge from a brewery wastewater treatment plant (Super Bock, Porto, Portugal) was used as inoculum, at a concentration of 10 $g \cdot L^{-1}$ in volatile solids (VS). Methanogens were previously inactivated by boiling the inoculum at 100 °C for 10 min. The reactor was operated at 37 °C, with a hydraulic retention time (HRT) of 7 days and the feeding pH was kept at 7. The substrate of the acidogenesis process consisted of a mixture of 20 $g \cdot L^{-1}$ (in COD) FW leachate and 20 $g \cdot L^{-1}$ (in COD) wastewater derived from the biodiesel industry. FW leachate was obtained by homogenizing the FW (Canteen of University of Minho, Braga, Portugal) with a blender, autoclaved at 121 °C, for 20 min, and in the end, was centrifuged at 15000 g for 15 min. Brine wastewater (224.3 $g \cdot L^{-1}$ of salinity and 8.3 $g \cdot L^{-1}$ of COD) from a fish-canning industry (A Poveira, Póvoa de Varzim, Portugal) was also added to the mixture to have a final salinity of 20 $g \cdot L^{-1}$.

The fermented medium was collected for 4 weeks, centrifuged, filtrated through a 0.45 μm membrane to remove the excess suspended solids, and preserved at – 20 °C. The characterization of VFAs-rich fermented medium (VFAs composition, chemical oxygen demand, total nitrogen, total organic carbon, reducing sugars, soluble protein, magnesium, phosphate, and potassium) was performed as described

in section 6.2.5 and is shown in Table 6.1. The VFAs-rich medium was sterilized at 121 °C for 15 min before being used as the substrate for *Y. lipolytica* cultures.

6.2.3. Erlenmeyer flasks experiments

The ability of *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 to grow in FW-derived VFAs medium and accumulate microbial lipids was firstly evaluated in batch cultures carried out in 500-mL baffled Erlenmeyer flasks containing 200 mL of FW-derived VFAs medium. Batch cultures on pure VFAs with the same VFAs composition were performed for comparative purposes. The culture medium of pure VFAs was supplemented with 2.04 $g \cdot L^{-1}$ of $(NH_4)_2SO_4$) to obtain a C/N mass ratio of 16 that is similar to that of FW-derived VFAs medium. Furthermore, an experiment in pure VFAs medium with a C/N mass ratio of 75 (by adding 0.43 $g \cdot L^{-1}$ of $(NH_4)_2SO_4$) was performed to evaluate the effect of the C/N ratio on yeast growth and lipids accumulation. The addition of 40 $g \cdot L^{-1}$ crude glycerol to FW-derived VFAs medium was also tested (C/N mass ratio of 30). Crude glycerol was supplied by Prio Energy – Prio Biocombustíveis, SA and was composed (w/w) of 82 % glycerol, 12 % water, 4.7 % NaCl, less than 0.01 % methanol, and 0.5 % organic matter (not glycerol). The initial pH of all culture media was adjusted to 6 and batch cultures were carried out at 27 °C and 170 rpm in an orbital incubator.

6.2.4. Bioreactor experiments

Bioreactor experiments were performed in a 2-L DASGIP Parallel Bioreactor System (Eppendorf, Germany) with 400 mL of working volume at a specific airflow rate of 3.5 vvm and an agitation rate of 550 rpm. During the cultivations, the pH was measured using a pH electrode (450-DPAS-SC-K851325-type, Mettler Toledo) and was automatically controlled at 6 by the addition of HCl 2 M and NaOH 2 M, while the temperature was maintained at 27 °C. Dissolved oxygen concentration was monitored with a polarographic oxygen probe (Inpro6820/12/320-type, Mettler Toledo).

6.2.4.1. Batch cultures

Batch cultures of *Y. lipolytica* NCYC 2904 were carried out to evaluate the yeast growth and lipid accumulation in three different culture media: (1) FW-derived VFAs medium (C/N mass ratio of 16); (2) Pure VFAs medium (C/N mass ratio of 75); and (3) crude glycerol medium (40 $g \cdot L^{-1}$ crude glycerol and 0.98 $g \cdot L^{-1}$ $(NH_4)_2SO_4$, C/N mass ratio of 75). The composition of media (1) and (2) was the same used in batch cultures carried out in Erlenmeyer flasks.

6.2.4.2. Two-stage batch cultures

In two-stage batch cultures (TSC), *Y. lipolytica* NCYC 2904 was grown for 34 h in FW-derived VFAs medium (1st stage), followed by the addition of crude glycerol (2nd stage). TSC-I was carried out with the addition of 40 g·L^{-1} crude glycerol to the culture medium after 34 h of yeast growth, corresponding to the time that all VFAs were consumed. In TSC-II experiments, the addition of two pulses of crude glycerol was studied: 40 g·L^{-1} crude glycerol at 34 h of yeast growth (1st pulse) and 25 g·L^{-1} crude glycerol after 24 h of 1st pulse (2nd pulse).

6.2.5. Analytical methods

Chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen (TN) and magnesium, phosphorus, and potassium concentration in VFAs-rich fermented medium were determined spectrophotometrically using the cuvette test kits LCK 014, LCK 387, LCK 338, LCK 326, LCK 350 and LCK 328 (Hach-Lange GmbH, Germany), respectively. Soluble protein was quantified by the Bradford method using bovine serum albumin as standard (Bradford 1976). Reducing sugars were measured by the DNS method using glucose as standard (Miller 1959). VFAs (acetic, propionic, iso-butyric, *n*-butyric, and *n*-valeric acids) and glycerol were analyzed by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (300mm x 7.8mm, 8 µm particle size) at 60 °C coupled to an ultra-violet (UV) and a refractive index (RI) detectors. The column temperature was maintained at 60 °C and sulfuric acid 5 mM at a flow rate of 0.7 mL·min^{-1} was used as the mobile phase.

The analysis of biomass concentration, microbial lipids and the fatty acid profile of microbial lipids were performed as described in the chapter 3.2.6.

6.2.6. Statistical analysis

One-way analysis of variance (ANOVA) was used for the statistical analysis of the data as described in chapter 3.2.8.

6.3. Results and Discussion

6.3.1. Erlenmeyer flasks experiments

The fermented medium obtained from the acidogenesis of organic wastes was predominantly composed of short-chain fatty acids (C2-C5) (Table 6.1). The iso-butyric (28 %, w/w), *n*-butyric (25 %, w/w) and acetic (24 %, w/w) acids were the most abundant VFAs, followed by propionic (11 %, w/w) and *n*-valeric (11 %, w/w) acids. Despite the fact that VFAs composition is affected by substrate and fermentation conditions, the prevalence of shorter VFAs is a common feature in the acidogenesis process using FW as feedstocks (Gao et al. 2017; Gao et al. 2020; Duarte et al. 2021).

Table 6.1- Composition of the fermented medium obtained by the acidogenesis of organic wastes: VFAs concentration, chemical oxygen demand (COD), total nitrogen (TN), total organic carbon (TOC), reducing sugars, soluble protein, magnesium, phosphorus and potassium concentration, pH, and carbon/nitrogen ratio (C/N).

Total VFAs ($g \cdot L^{-1}$)	13.90 ± 0.78
Acetic acid ($g \cdot L^{-1}$)	3.33 ± 0.24
Propionic acid ($g \cdot L^{-1}$)	1.53 ± 0.21
iso-Butyric acid ($g \cdot L^{-1}$)	3.83 ± 0.21
***n*-Butyric acid ($g \cdot L^{-1}$)**	3.43 ± 0.27
***n*-Valeric acid ($g \cdot L^{-1}$)**	1.47 ± 0.06
COD ($g \cdot L^{-1}$)	31.02 ± 0.16
TN ($g \cdot L^{-1}$)	1.041 ± 0.001
TOC ($g \cdot L^{-1}$)	16.55 ± 2.11
Magnesium ($mg \cdot L^{-1}$)	36 ± 1
Phosphorus ($mg \cdot L^{-1}$)	193 ± 7
Potassium ($mg \cdot L^{-1}$)	1520 ± 339
Reducing sugars ($mg \cdot L^{-1}$)	310 ± 15
Soluble protein ($mg \cdot L^{-1}$)	ND
pH	5.8
C/N	16

ND – not detected

Yarrowia lipolytica W29 and *Y. lipolytica* NCYC 2904 are already known for their ability to use pure VFAs (acetic, propionic, and *n*-butyric acid) as carbon and energy sources (Pereira et al., 2021). In the present work, the cellular growth and microbial lipids production by both yeast strains were evaluated in a VFAs-rich fermented medium obtained by the acidogenic process of FW. Additionally, a pure VFAs medium with the same VFAs composition and nitrogen concentration of FW-derived VFAs was carried out (pure VFAs medium with C/N 16), as well as cultures under nitrogen limitation (pure VFAs medium with C/N 75). *Yarrowia lipolytica* W29 and *Y. lipolytica* NCYC 2904 were able to grow in FW-derived VFAs and no significant differences in biomass concentration and biomass yield were observed between the FW-derived VFAs and pure VFAs (C/N 16) experiments by W29 strain (Figure 6.1A and 6.1B, Table 6.2), indicating that this yeast strain is able to grow in the complex medium rich in VFAs obtained by acidogenesis of FW. Other studies also confirmed the suitability of using VFAs generated from anaerobic fermentation of organic wastes as carbon sources for *Y. lipolytica* biomass production (Gao et al. 2017; Gao et al. 2020; Llamas et al. 2020c; Morales-Palomo et al. 2022). *Yarrowia lipolytica* W29 cultures reached 1.6- and 1.3-fold higher biomass concentration and biomass yield, respectively, in higher nitrogen concentration conditions (pure VFAs with C/N 16) than in pure VFAs with C/N 75 (Figure 6.1B and 6.1C, Table 6.2). Since nitrogen is an essential nutrient for protein and nucleic acid synthesis, the higher availability of nitrogen (low C/N ratio) boosted biomass growth (Huang et al. 2016; Hapeta et al. 2020).

Yarrowia lipolytica NCYC 2904 was able to grow in all culture media tested (Figure 6.1D, 6.1E, and 6.1F), but the highest biomass concentration and biomass yield were obtained in FW-derived VFAs (Table 6.2). Biomass production in FW-derived VFAs was 3-fold higher than that obtained in pure VFAs with a C/N ratio of 16 (Figure 6.1D and 6.1E), suggesting that the consumption of the micro-nutrients of the fermented FW enhanced *Y. lipolytica* NCYC 2904 growth. In fact, 65 % of magnesium and 43 % of phosphorus in VFAs-rich fermented medium were consumed after 72 h of cultivation. Furthermore, according to the stoichiometric calculation based on the VFAs composition of fermented FW (Table 6.1), the COD of VFAs was 19.6 $g \cdot L^{-1}$, which corresponds to 63.2 % of the overall COD obtained from FW-derived VFAs medium. The remaining 36.8 % of COD represents other organic matter present in the medium that might have been used for *Y. lipolytica* NCYC 2904 growth. However, Morales-Palomo et al. (2002) reported that additional organic matter was not consumed by *Y. lipolytica* ACA DC 50109 and stated that the consumption of microelements such as sodium, phosphorus, magnesium, and potassium present on fermented FW could be responsible for improved yeast growth. Contrarily to the W29 strain, the nitrogen concentration in pure VFAs media had no effect on *Y. lipolytica* NCYC 2904 growth profile and biomass yield (Figure 6.1E and 6.1F, Table 6.2).

All VFAs were completely consumed by *Y. lipolytica* W29 after 34 h of yeast cultivation in FW-derived VFAs (Figure 6.1A), while 98 % and 79 % of VFAs were consumed at the end of the experiments (72 h) in pure VFAs with C/N 16 (Figure 6.1B) and C/N 75 (Figure 6.1C), respectively. In fact, the total VFAs uptake rate (Table 6.1) was approximately 3 times higher in FW-derived VFAs than in media with pure VFAs. In *Y. lipolytica* ACA DC 5010 cultures, all VFAs in the fermented medium were consumed after 32 h, but 144 h was required to consume 96 % of VFAs in a synthetic medium (Llamas et al. 2020c). Similar to the W29 strain, *Y. lipolytica* NCYC 2904 consumed all VFAs after 34 h of cultivation in FW-derived VFAs medium. The total VFAs uptake rate in pure VFAs media was approximately 7-fold lower than that in FW-derived VFAs medium, and only 36 % and 39 % of VFAs in pure VFAs with C/N 16 and C/N 75 were consumed by NCYC 2904 strain at 72 h of yeast culture, respectively. No significant differences in total VFAs uptake rates were achieved by varying the C/N ratio in the pure VFAs medium (Table 6.2), suggesting that the C/N ratio did not affect VFAs utilization by both strains. Regardless of the culture medium, a preference for acetic acid over other VFAs was observed, which is in accordance with previous studies that reported that *Y. lipolytica* metabolizes faster acetic acid than other VFAs (Gao et al. 2017; Pereira et al. 2021).

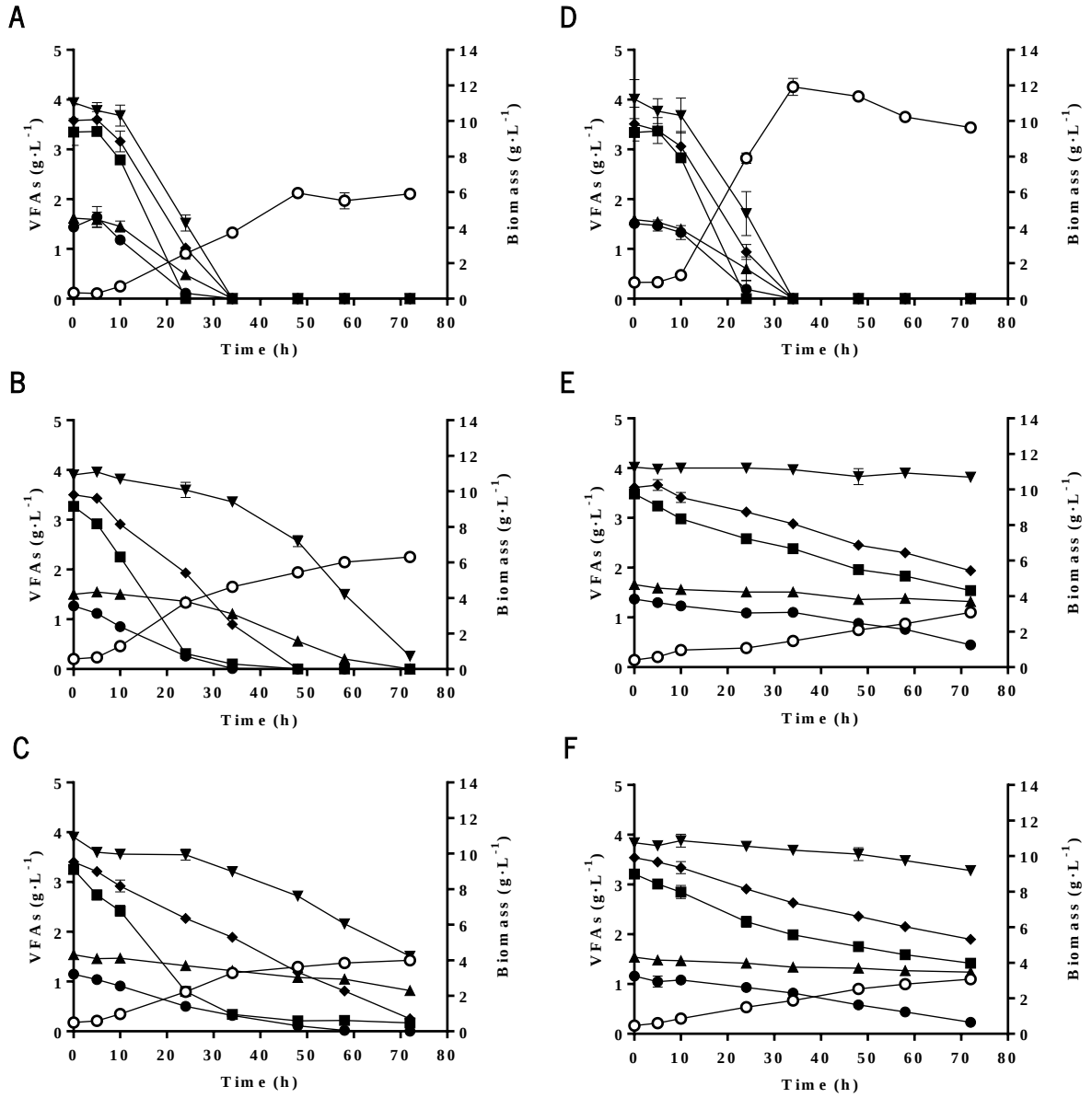

Figure 6.1 - Time course of cellular growth (○) and VFAs (■, acetic acid; ▲, propionic acid; ▼, iso-butyric acid; ◆, n-butyric acid; ●, n-valeric acid) utilization by *Y. lipolytica* W29 (left column) and *Y. lipolytica* NCYC 2904 (right column) in batch cultures carried out in Erlenmeyer flasks with FW-derived VFAs (A and D), pure VFAs (C/N 16) (B and E) and pure VFAs (C/N 75) (C and F).

Table 6.2 - Values of biomass yield ($Y_{X/S}$), lipids yield ($Y_{L/S}$), lipids content, lipids concentration, and total VFAs uptake rate (R_{VFAs}) obtained in *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 batch cultures carried out in Erlenmeyer flasks. Data are average ± standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences ($p \geq 0.05$).

Y. lipolytica strain	Culture medium	C/N	$Y_{X/S}$ ($g \cdot g^{-1}$)	$Y_{L/S}$ ($g \cdot g^{-1}$)	Lipids content (%, w/w)	Lipids concentration ($g \cdot L^{-1}$)	R_{VFAs} ($g \cdot L^{-1} \cdot h^{-1}$)
W29	FW-derived VFAs	16	0.41 ± 0.01 [a]	0.03 ± 0.01 [a]	7.2 ± 0.4 [a]	0.4 ± 0.1 [a]	0.52 ± 0.03 [a]
	Pure VFAs	16	0.43 ± 0.01 [a]	0.07 ± 0.01 [b]	16.0 ± 3 [b]	1.0 ± 0.2 [b]	0.194 ± 0.001 [b]
	Pure VFAs	75	0.334 ± 0.001 [b]	0.08 ± 0.01 [b]	20 ± 3 [b]	0.8 ± 0.1 [b]	0.145 ± 0.001 [b]
NCYC 2904	FW-derived VFAs	16	0.61 ± 0.01 [c]	0.024 ± 0.002 [a]	3.6 ± 0.6 [a]	0.35 ± 0.04 [a]	0.50 ± 0.02 [a]
	Pure VFAs	16	0.53 ± 0.01 [d]	0.16 ± 0.01 [c]	27 ± 2 [c]	0.83 ± 0.05 [b]	0.069 ± 0.001 [c]
	Pure VFAs	75	0.50 ± 0.01 [d]	0.25 ± 0.02 [d]	42 ± 4 [d]	1.30 ± 0.04 [c]	0.073 ± 0.001 [c]

Both yeast strains accumulated less intracellular lipids in FW-derived VFAs medium than in pure VFAs media (Table 6.2). The high protein content of food waste may increase the amount of ammonium nitrogen in VFAs-rich fermented medium and consequently lead to low C/N ratios. In this study, the FW-derived VFAs medium had a C/N ratio of 16 that, according to the literature, favors yeast cell proliferation instead of lipid synthesis (Papanikolaou and Aggelis, 2011). Moreover, with *Y. lipolytica* W29 cultures the results of intracellular lipids content, lipids yield, and lipids concentration were 2-fold higher when cells were cultivated in pure VFAs medium with C/N 16 than in FW-derived VFA medium (Table 6.2). Improvements of 8-, 7-, and 2-fold of lipids content, lipids yield, and lipids concentration, respectively, were attained in *Y. lipolytica* NCYC 2904 batch cultures with pure VFAs (C/N 16), compared to those obtained in FW-derided VFAs (Table 6.2). Despite having the same VFAs and nitrogen concentration, the micro-nutrient (P, Mg, and K) absence in the pure VFAs medium (C/N 16) favored lipids accumulation. By contrast, the presence of these micro-nutrients in FW-derived VFAs medium, even in small amounts, had a negative effect on lipids accumulation. In nutrient-limited conditions, such as magnesium and phosphorus, high lipids content can be accumulated by *Y. lipolytica* strains (Bellou et al. 2016; Wierzchowska et al. 2021).

The highest lipids content was obtained in the pure VFAs with a C/N ratio of 75 for the NCYC 2904 strain but this was also the best condition for the highest lipids accumulation in strain W29. The maximum lipids content of 20 % (w/w) and 42 % (w/w) attained for *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 cultures in pure VFAs (C/N 75) were 3- and 12-fold higher than that in FW-derived VFAs, respectively. Although it has been reported that the accumulation of microbial lipids by *Y. lipolytica* from most VFAs occurs via *ex novo* lipid biosynthesis pathway, whereas acetic acid is uptaken via *de novo* pathways (Morales-Palomo et al., 2022), the results obtained herein demonstrated that the C/N ratio had an important role in microbial lipids production, particularly by *Y. lipolytica* NCYC 2904. For *Y. lipolytica* NCYC 2904, a 2-fold improvement in intracellular lipids production was obtained with the increase of the C/N ratio from 16 to 75 in pure VFAs. It should be noticed that the C/N ratio in pure VFAs media had different effects depending on the yeast strain. While in *Y. lipolytica* W29 cultures the C/N ratio affected the yeast growth, in *Y. lipolytica* NCYC 2904 the C/N ratio affected lipids biosynthesis. These results supported the utilization of VFAs-rich fermented medium for cellular growth, but not for lipids production. Our previous work demonstrated an enhancement in *Y. lipoly*tica growth and intracellular lipids production from pure VFAs (a mixture of acetic, propionic, and butyric acids) by supplementing the pure VFAs medium with pure glycerol (Pereira et al. 2021). Herein, the addition of 40 $g \cdot L^{-1}$ crude glycerol to FW-derived VFAs medium was tested as an attempt to improve lipids production. Crude glycerol is a by-

product of biodiesel production, in which 1 kg of glycerol is generated from 10 kg of biodiesel produced (Lopes et al. 2022). Despite the decrease in global biomass yield (cell mass per mass of total substrates, VFAs, and glycerol consumed) with the addition of crude glycerol to FW-derived VFAs (0.11 $g \cdot g^{-1} \pm 0.01$ $g \cdot g^{-1}$ for W29 strain and to 0.37 $g \cdot g^{-1} \pm 0.01$ $g \cdot g^{-1}$ for NCYC 2904 strain), similar final biomass concentration was attained in FW-derived VFAs (Figure 6.1A, 6.1D and 6.2).

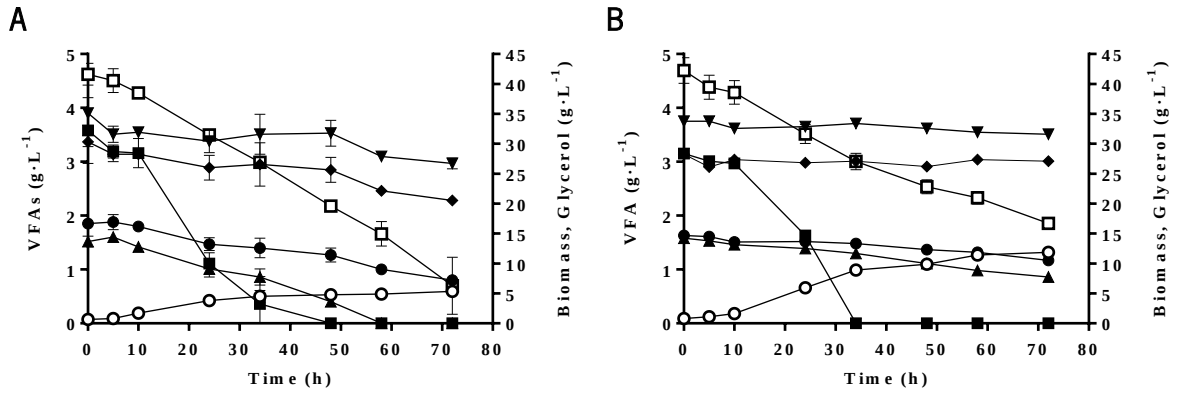

Figure 6.2 - Time course of cellular growth (○), VFAs (■, acetic acid; ▲, propionic acid; ▼, iso-butyric acid; ◆, n-butyric acid; ●, n-valeric acid) and glycerol (□) utilization by *Y. lipolytica* W29 (A) and *Y. lipolytica* NCYC 2904 (B) in batch cultures carried out in Erlenmeyer flasks with FW-derived VFAs supplemented with 40 $g \cdot L^{-1}$ crude glycerol.

The addition of crude glycerol changed the VFAs consumption profile of both yeast strains. The total VFAs uptake rate of *Y. lipolytica* W29 (0.118 $g \cdot L^{-1} \cdot h^{-1} \pm 0.008$ $g \cdot L^{-1} \cdot h^{-1}$) and *Y. lipolytica* NCYC 2904 (0.072 $g \cdot L^{-1} \cdot h^{-1} \pm 0.008$ $g \cdot L^{-1} \cdot h^{-1}$) were 4- and 7- fold lower, respectively, than those attained in FW-derived VFAs cultures, suggesting that crude glycerol had catabolic repression on VFAs uptake. Though yeasts did not consume all glycerol during the experiments time, glycerol uptake rates (0.49 $g \cdot L^{-1} \cdot h^{-1} \pm 0.03$ $g \cdot L^{-1} \cdot h^{-1}$ for W29 strain and 0.36 $g \cdot L^{-1} \cdot h^{-1} \pm 0.02$ $g \cdot L^{-1} \cdot h^{-1}$ for NCYC 2904 strain) were considerably higher than the total VFAs uptake rates. Furthermore, a sequential (diauxic) utilization of different VFAs was observed for both strains. The assimilation of iso-butyric and *n*-butyric acids was decreased, or even absent (Figure 6.2) in the presence of glycerol. Using glucose as a co-substrate in a pure VFAs medium, Pereira et al. (2022b) observed that the VFAs uptake rate was not affected by the addition of glucose and a preference of *Y. lipolytica* to assimilate VFAs over glucose was observed.

Some works in the literature demonstrated the potential of using crude glycerol as feedstock for lipid biosynthesis by *Y. lipolytica* (Dobrowolski et al. 2016; Kumar et al. 2021). The presence of glycerol led

to an enhancement in lipids content (13 %± 2 % for W29 strain and 6.8 %± 0.5 % for NCYC 2904 strain) and lipids concentration (0.7 $g{\cdot}L^{-1}$ ± 0.1 $g{\cdot}L^{-1}$ for W29 strain and 0.81 $g{\cdot}L^{-1}$ ± 0.06 $g{\cdot}L^{-1}$ for NCYC 2904 strain), but lipids yield (0.016 $g{\cdot}g^{-1}$ ± 0.003 $g{\cdot}g^{-1}$ for W29 strain and 0.027 $g{\cdot}g^{-1}$ ± 0.001 $g{\cdot}g^{-1}$ for NCYC 2904 strain) were not improved by the addition of the co-substrate. Though a 2-fold increase in lipids content was observed, lipids accumulated remained low, pointing that the additional substrate was channeled towards the production of other metabolites, or even for cellular growth, instead of microbial lipids. In fact, inside the cell, glycerol is phosphorylated into glycerol-3-phosphate and then oxidized to 3-P-glyceraldehyde, which can enter the glycolytic pathway and be transformed into acetyl-CoA. Acetyl-CoA is a precursor for a variety of metabolites through the TCA cycle (Lopes et al. 2022).

The results from Erlenmeyer flask experiments demonstrated the feasibility of *Y. lipolytica* W29 and *Y. lipolytica* NCYC 2904 using VFAs-rich fermented medium obtained in the acidogenic process of FW as carbon source, with a remarkable potential of biomass production by NCYC 2904 strain.

6.3.2. Bioreactor experiments

6.3.2.1. *Yarrowia lipolytica* NCYC 2904 batch cultures

The results described above demonstrated that FW-derived VFAs can be used as a substrate for *Y. lipolytica* NCYC 2904 growth. Moreover, in our previous works (Pereira et al., 2022b, 2021) it was demonstrated the potential of this yeast strain to synthesize lipids from a mixture of pure VFAs (acetic, propionic, and butyric acids). Thus, *Y. lipolytica* NCYC 2904 batch cultures on FW-derived VFAs were carried out in a laboratory-scale STR. Additionally, batch cultures with pure VFAs and crude glycerol were carried out in order to compare the yeast growth and lipids accumulation.

The highest cellular concentration was attained in FW-derived VFAs and crude glycerol (Figure.6.3A and 6.3 C), but in FW-derived VFAs a higher biomass yield was obtained (Table 6.3), confirming the positive effect of VFAs-rich fermented medium on *Y. lipolytica* NCYC 2904 growth. All VFAs were consumed by the yeast after 34 h of cultivation in FW-derived VFAs as observed in Erlenmeyer flask experiments. The dissolved oxygen concentration dropped to zero in the first 24 h of cultivation, increased during the stationary phase of growth, and remained above 80 % until the end of experiments (Figure 6.3D).

The up-scaling of the batch from the Erlenmeyer flask to the bioreactor of *Y. lipolytica* NCYC 29054 cultures in pure VFAs (C/N 75) led to an approximately 2- and 3- fold improvement in yeast growth and total VFAs uptake rate, respectively (Figure 6.3B and Table 6.3). Unlike what was observed in Erlenmeyer flask experiments (Figure 6.1F), all VFAs were consumed at the end of the experiments, with the

exception of iso-butyric acid (1.6 g·L^{-1} remained in the culture media) (Figure 6.3B). Although no differences were observed in the content of microbial lipids accumulated by the yeast, the increase of final biomass led to a 1.5-fold enhancement of lipids concentration when compared with Erlenmeyer flask experiments. The dissolved oxygen concentration in the VFAs-based medium remained above 70 % of saturation. Dissolved oxygen concentration, under non-limiting conditions, can be an important factor in lipids accumulation since it is an electron acceptor in the yeast respiratory chain, in which is produced NAPDH that can be further utilized for lipids synthesis (Zhang et al. 2019b). Bellou et al. (2014) reported that high dissolved oxygen concentration favored lipid synthesis by *Y. lipolytica* due to the increase in the activity of enzymes involved in lipid synthesis, such as ATP-citrate lyase and malic enzyme.

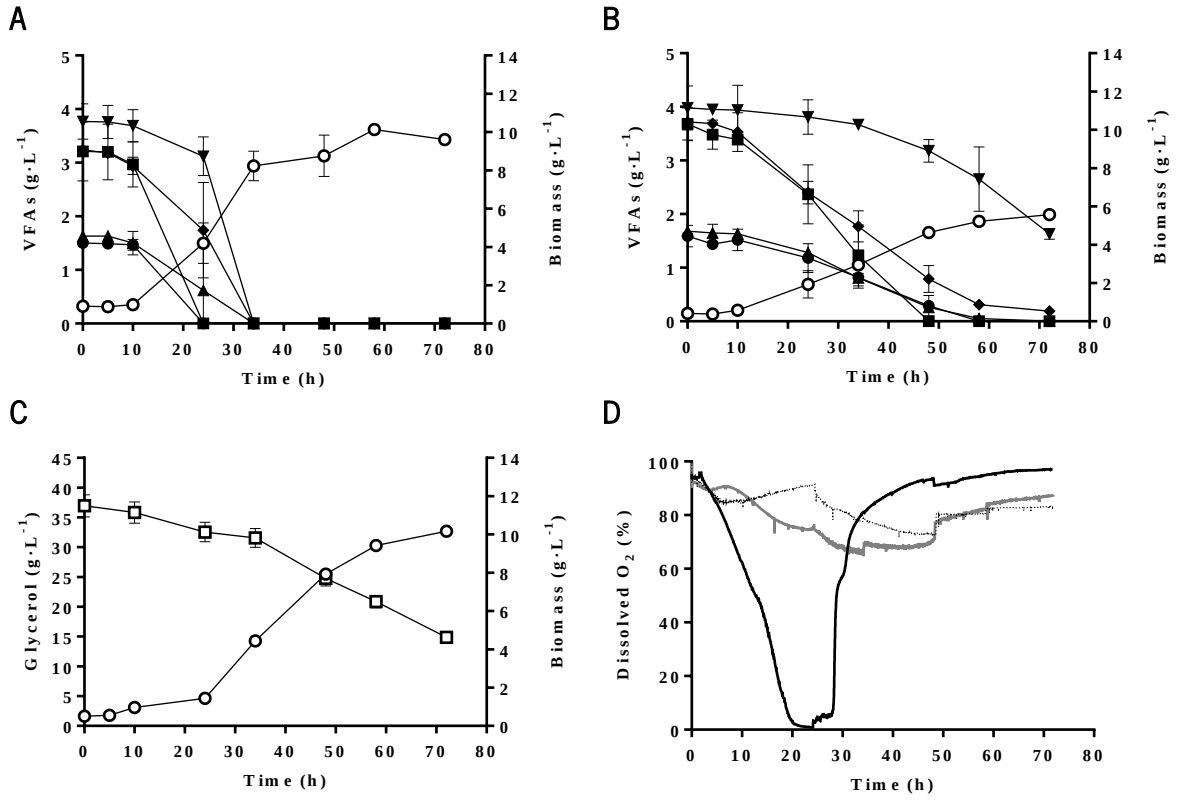

Figure 6.3 - Time course of cellular growth (○), VFAs (■, acetic acid; ▲, propionic acid; ▼, iso-butyric acid; ◆, n-butyric acid; ●, n-valeric acid) and glycerol (□) utilization by *Y. lipolytica* NCYC 2904 in batch cultures carried out in STR bioreactor with FW-derived VFAs (A), pure VFAs (B) and crude glycerol (C). Dissolved oxygen concentration (% of saturation) (D) obtained in FW-derived VFAs (black line), pure VFAs (grey line), and crude glycerol (black dashed line).

Table 6.3 - Values of biomass yield ($Y_{X/S}$), lipids yield ($Y_{L/S}$), lipids content, lipids concentration, and total VFAs uptake rate (R_{VFAs}) obtained in *Y. lipolytica* NCYC 2904 batch cultures carried out in STR bioreactor. Data are average ± standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences ($p \geq 0.05$).

Culture medium	$Y_{X/S}$ ($g \cdot g^{-1}$)	$Y_{L/S}$ ($g \cdot g^{-1}$)	Lipids content (%, w/w)	Lipids concentration ($g \cdot L^{-1}$)	R_{VFAs} ($g \cdot L^{-1} \cdot h^{-1}$)
FW-derived VFAs	0.65 ± 0.04 [a]	0.037 ± 0.003 [a]	5.2 ± 0.5 [a]	0.50 ± 0.05 [a]	0.47 ± 0.05 [a]
Pure VFAs	0.40 ± 0.01 [b]	0.145 ± 0.003 [b]	42 ± 5 [b]	1.9 ± 0.1 [b]	0.197 ± 0.004 [b]
Crude glycerol	0.48 ± 0.02 [b]	0.175 ± 0.004 [c]	38 ± 2 [b]	3.9 ± 0.2 [c]	-

Crude glycerol has been considered a cheap raw material for biotechnological processes and, in nitrogen-limited conditions, high amounts of microbial lipids were produced by *Y. lipolytica* strains (Dobrowolski et al. 2016). The final biomass concentration obtained in the crude glycerol was similar to that attained in FW-derived VFAs and 2-fold higher than in the pure VFAs (Figure 6.3). Although the content of microbial lipids accumulated by *Y. lipolytica* NCYC 2904 in the glycerol medium is similar to that reached in pure VFAs, the lipids yield and lipids concentration were 1.4- and 2- fold higher, respectively (Table 6.3). Additionally, the lipids yield, lipids content and lipids concentration obtained in glycerol were 5-, 7- and 8- fold higher than those obtained with FW-derived VFAs, respectively (Table 6.3). As observed with pure VFAs, the dissolved oxygen concentration in the glycerol medium remained at non-limiting concentration values.

On whole, FW-derived VFAs were suitable for *Y. lipolytica* NCYC 2904 growth instead of lipids accumulation. On the other hand, high amounts of microbial lipids were produced by yeast using crude glycerol as a carbon source. Thus, it would be interesting to test a two-stage batch culture to boost lipid synthesis by *Y. lipolytica* NCYC 2904, using a VFAs-rich fermented medium for cellular growth and crude glycerol for lipids accumulation.

6.3.2.2. Two-stage batch cultures of *Y. lipolytica* NCYC 2904

Traditional batch cultivations face difficulties in optimizing culture conditions for simultaneous growth and lipids production, since nitrogen-limited media is suitable for lipid synthesis, while nitrogen-rich media promote cell proliferation. Two-stage batch cultures, in which cell proliferation (1st phase – growth phase)

is followed by the lipid synthesis (2nd phase – lipogenic phase), proved to be advantageous for increasing lipid production (Karamerou and Webb 2019; Pereira et al. 2021). Although without an improvement in biomass yield, our previous work demonstrated a 3- fold enhancement in lipids yield by *Y. lipolytica* NCYC 2904 by using a two-stage batch culture – yeast growth in glucose, followed by the addition of 18 $g \cdot L^{-1}$ pure VFAs, comparatively to batch cultures (Pereira et al. 2022b). Other studies have shown the potential of employing this cultivation strategy, adding VFAs to cells grown on glucose or glycerol (Fontanille et al. 2012; Chatterjee and Mohan 2020; Qian et al. 2021). Comparatively to *Y. lipolytica* MUCL 28849 batch cultures, higher biomass and lipids yields (4.4 times) were achieved in two-stage fed-batch strategy, in which the lipogenic phase in pure butyrate was preceded by growth in glycerol (Fontanille et al. 2012). A 1.6-fold improvement in lipids yield was attained in *Cryptococcus curvatus* MTCC 269 cultures growing in glucose followed by the addition of pure VFAs mixture or VFAs rich acidogenic effluent derived from the renewable biohydrogen production process. By contrast, biomass yield was not affected by this operation mode (Chatterjee and Mohan 2020). The use of a two-stage automated fed-batch strategy, in which growth of *A. porosum* in glucose was followed by the addition of pure VFAs or corn stover hydrolysate containing VFAs, resulted in an increase in biomass and lipids yield (Qian et al. 2021).

Considering the potential of using FW-derived VFAs for cellular growth and the great content of microbial lipids obtained in crude glycerol medium (Figure 6.3, Table 6.3), a TSC strategy (growth in FW-derived VFAs – 1st stage – followed by the addition of crude glycerol – 2nd stage) was studied as an attempt to enhance microbial lipids production. In the first approach, *Y. lipolytica* NCYC cells grew in FW-derived VFAs for 34 h, followed by the addition of 40 $g \cdot L^{-1}$ crude glycerol (TSC-I). In the second approach, two pulses of crude glycerol (40 $g \cdot L^{-1}$ and 25 $g \cdot L^{-1}$ at 34 h and 58 h of yeast cultivation) were added (TSC-II). To our best knowledge, this is the first time that this TSC strategy is studied for lipids production by *Y. lipolytica* NCYC 2904 using FW-derived VFAs and crude glycerol as substrates. No improvement in biomass yield was attained with the TSC cultures (Table 6.4) relative to batch cultures of *Y. lipolytica* NCYC 2904 in FW-derived VFAs and glycerol medium (Table 6.3), however, a higher cellular density was reached in TSC cultures (approximately 32 $g \cdot L^{-1}$ biomass). Regardless of TSC approaches, significant growth was observed during the first 24 h after the addition of 40 $g \cdot L^{-1}$ crude glycerol. In this period, the glycerol uptake rate (1.03 $g \cdot L^{-1} \cdot h^{-1} \pm 0.05$ $g \cdot L^{-1} \cdot h^{-1}$) was considerably higher than that obtained in batch cultures, due to the increased cellular concentration of the culture. Moreover, it is noted that glycerol was consumed faster than that in batch cultures in Erlenmeyer flask experiments since, at the moment of addition of VFAs, all VFAs had already been consumed, while in Erlenmeyer flask experiments, glycerol was consumed simultaneously with VFAs. With the addition of the second pulse of glycerol (TSC-II), no

significant cellular growth was observed, despite the glycerol being completely consumed at a similar uptake rate (0.91 $g \cdot L^{-1} \cdot h^{-1} \pm 0.09\ g \cdot L^{-1} \cdot h^{-1}$) than that observed after the 1st pulse. This suggests that glycerol was channeled towards cellular maintenance, lipids synthesis, or other metabolites instead biomass production.

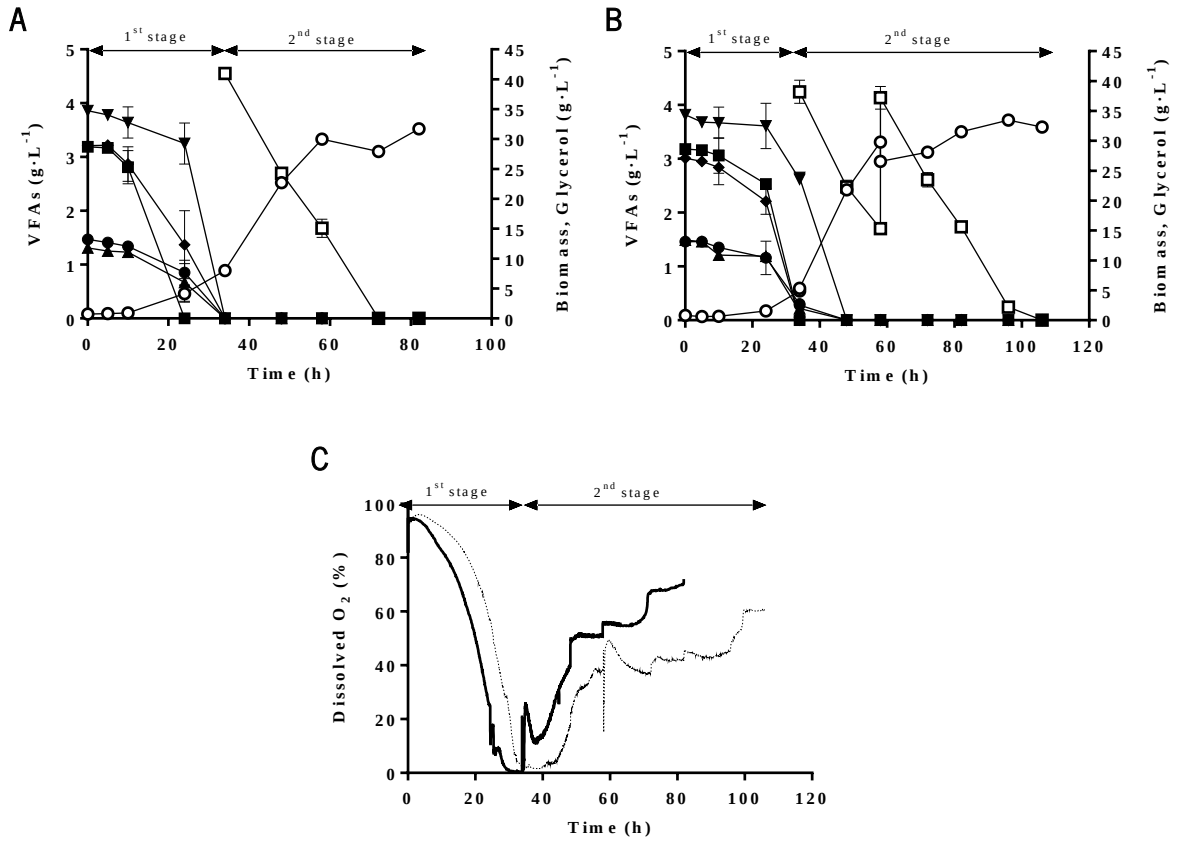

Figure 6.4 - Time course of cellular growth (○), VFAs (■, acetic acid; ▲, propionic acid; ▼, iso-butyric acid; ◆, n-butyric acid; ●, n-valeric acid) and glycerol (□) utilization by *Y. lipolytica* NCYC 2904 in two-stage batch cultures carried out in STR bioreactor: TSC-I (A) and TSC-II (B). Dissolved oxygen concentration (% of saturation) obtained in TSC-I (black line) and TSC-II (black dashed line) (C).

Table 6.4 - Values of global biomass yield ($Y_{X/S}$), lipids yield ($Y_{L/S}$), lipids content, and lipids concentration obtained at the end of *Y. lipolytica* NCYC 2904 TSC cultures carried out in STR bioreactor. Data are average ± standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences ($p \geq 0.05$).

TSC culture	$Y_{X/S}$ ($g \cdot g^{-1}$)	$Y_{L/S}$ ($g \cdot g^{-1}$)	Lipids content (%, w/w)	Lipids concentration ($g \cdot L^{-1}$)
TSC-I	0.57 ± 0.01 [a]	0.139 ± 0.002 [a]	22 ± 1 [a]	7.0 ± 0.5 [a]
TSC-II	0.432 ± 0.007 [b]	0.153 ± 0.005 [a]	35 ± 2 [b]	11.2 ± 0.6 [b]

$Y_{X/S}$ and $Y_{L/S}$ were calculated as cell mass and lipid mass, respectively, per mass of total substrates (VFAs and glycerol) consumed.

Two-stage batch cultures were demonstrated to be an effective strategy to promote yeast growth and microbial lipids production from FW-derived VFAs and crude glycerol. The highest lipids production was obtained in TSC-II, being the maximum lipids content and lipids concentration in this condition approximately 2- fold higher than those attained in TSC-I (Table 6.4). This enhancement can be correlated with the limitation of nitrogen and excess carbon source (crude glycerol) in the culture media after the 2nd pulse. The addition of the 2nd pulse of crude glycerol led to an increase in the C/N ratio, favoring the production of lipids, while the nitrogen-limitation decreased cell proliferation. For the accumulation of microbial lipids by *Y. lipolytica* from hydrophilic substrates, such as glycerol, the C/N ratio is the most important factor affecting lipids accumulation by *Y. lipolytica*, since the *de novo* biosynthesis pathway is induced by nitrogen limitation and excess of carbon source (Papanikolaou and Aggelis 2011b). It is worth noticing that there was a 10- and a 5-fold improvement in lipids content by *Y. lipolytica* in TSC-II compared with those obtained in batch cultures with FW-derived VFAs and FW-derived VFAs supplemented with crude glycerol (Erlenmeyer flasks experiments), respectively. The TSC-II was demonstrated to be an effective strategy to boost lipids synthesis by *Y. lipolytica* NCYC 2904. In fact, approximately 22- and 3-fold enhancement in microbial lipids concentration was attained for *Y. lipolytica* NCYC 2904 cultures when compared to batch cultures with FW-derived VFAs and crude glycerol (bioreactor experiments), respectively, due to the high final cellular concentration obtained in TSC-II cultures. Furthermore, it is noteworthy a 32- and 14- fold improvement in lipids concentration with the TCS-II approach compared to that obtained in FW-derived VFAs medium and FW-derived VFAs medium supplemented with crude glycerol (Erlenmeyer flasks experiments), respectively. As observed in batch cultures, lipids accumulation was favored in the dissolved oxygen at non-limiting concentration. In both TSC cultures, the dissolved

oxygen concentration in the medium increased during the lipogenic phase in both TSC cultures, remaining above 40 % (Figure 6.4C).

Microbial lipids produced at the end of the *Y. lipolytica* NCYC 2904 cultures in TSC-II (the condition where a higher amount of lipids was obtained) were predominantly composed of oleic acid (52.6 % ± 0.5 %), followed by palmitic (12.2 % ± 0.2 %), palmitoleic (12.1 % ± 0.2 %), linoleic (11.5 % ± 0.1 %), and stearic (7.2 % ± 0.4 %) acids. Although in low amounts, odd-chain fatty acids, namely heptadecenoic (3.5 % ± 0.1 %) and margaric (0.90 % ± 0.04 %) acids were also produced, owing to the presence of propionic acid in FW-derived VFAs medium (Llamas et al. 2020a). Odd-chain fatty acids have attracted great attention from the scientific community since they can be used in a variety of pharmaceutical and industrial applications (Park et al. 2018). The total amount of unsaturated fatty acids was higher than 80 %, showing the high similarity of these microbial lipids with vegetable oils, traditionally used as raw feedstocks in the biodiesel industry. Thus, microbial lipids produced by *Y. lipolytica* NCYC 2904 from FW-derived VFAs and crude glycerol, using a TSC strategy, proved to be highly suitable for biodiesel production.

The present study demonstrates the great value of food waste and crude glycerol as low-cost nutrient sources for biomass and lipid production. In this sense, microbial lipids production through the valorization of low-valued feedstock is a highly promising strategy to guarantee, simultaneously, obtaining a value-added product and the full utilization of wastes and by-products without further waste disposal, under the circular economy guidelines.

6.4. Conclusions

The results demonstrate the potential application of *Y. lipolytica* for biomass production using the VFAs-rich fermented medium obtained in the acidogenic process of FW. A TSC strategy with a growth phase in FW-derived VFAs, followed by the addition of two pulses of crude glycerol, was clearly the best strategy to produce a high amount of lipids. Lipids produced by *Y. lipolytica* NCYC 2904 were mostly composed of unsaturated fatty acids, making these lipids a plausible feedstock in the biodiesel industry. Therefore, this bioprocess is an effective and sustainable strategy for lipids production using low-cost feedstocks in an integrated biorefinery framework.

CHAPTER 7

From crude glycerol and volatile fatty acids to biodiesel and other bioproducts using by *Yarrowia lipolytica* NCYC 2904 as cell factory

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This chapter is based on the following original research article:

Pereira, A. S., Lopes, M., Belo, I. (2023) From crude glycerol and volatile fatty acids to biodiesel and other bioproducts using *Yarrowia lipolytica* NCYC 2904 as a cell factory. Sustainable Energy and Fuels, 7: 4687-4696. https://doi.org/10.1039/D3SE00682D

7.1 Introduction

Over the last decades, biodiesel has been promoted as a renewable and environmentally preferable option to conventional fossil fuels. Currently, about 73 % of biodiesel is produced from pure vegetable oils (31 % palm oil, 24 % soybean oil, and 14 % rapeseed oil) and waste cooking oils (21 %) (OECD 2022). However, the use of these feedstocks has become controversial due to competition with food production and the diverting of land to biofuel production. Growing demand for crops of vegetable oils leads to an increase in the use of fertilizers and increases greenhouses gases emissions (Jeswani et al. 2020). Microbial lipids produced by oleaginous microorganisms, is considered a promising feedstock for sustainable biodiesel production owing to their composition of fatty acids similar to those of vegetable oils. Additionally, microbial lipids have some advantages over vegetable oils such as non-dependence on land and climate conditions, and can be produced from a wide spectrum of feedstocks with a short period of production (Vasconcelos et al. 2019; Szczepańska et al. 2021).

The oleaginous yeast *Yarrowia lipolytica* is considered a yeast model for microbial lipids production owing to its ability to accumulate high amounts of lipids from a wide range of substrates, making this yeast a sustainable source of lipids for the biodiesel industry (Caporusso et al. 2021; Lopes et al. 2022). Moreover, this non-conventional yeast has the remarkable ability to assimilate carbon sources from various agro-industrial wastes or by-products, including sugars from lignocellulosic biomass hydrolysates (Vasaki et al. 2022), volatile fatty acids (VFAs) obtained by acidogenic fermentation of organic wastes, crude glycerol from biodiesel industry (Pereira et al. 2023), and fatty acids derived from olive mill wastewater (Lopes et al. 2009a), animal fat (Lopes et al. 2018) and waste cooking oils (Lopes et al. 2019). Among the wide range of carbon sources utilized by *Y. lipolytica*, our previous work (Pereira et al. 2023) demonstrated the feasibility of using crude glycerol and VFAs for lipids production. However, the main focus was the use of VFAs from the beginning of the culture time, and glycerol was used as a co-substrate. However, due to the availability of crude glycerol its use as a main substrate for high-cell density cultures and intracellular lipids accumulation is of utmost interest.

Crude glycerol is the main by-product of the biodiesel industry since about 10 % (v/v) of crude glycerol is generated from the amount of biodiesel produced (Monteiro et al. 2018). The global production of biodiesel is projected to reach 53.2 billion liters by 2026 (IEA, 2021), implying that a large amount of crude glycerol will be available, arising opportunities for the development of innovative processes using this by-product. Volatile fatty acids are short-chain fatty acids (C2-C6) obtained during the acidogenic fermentation of a variety of wastes rich in organic matter, such as food waste. Among VFAs produced by

acidogenic fermentation, 80 % of VFAs correspond to acetic, propionic, and butyric acids (Gameiro et al. 2016; Gao et al. 2017; Moretto et al. 2019a; Gao et al. 2020; Duarte et al. 2021; Pereira et al. 2023). Besides being a yeast model for lipids production, *Y. lipolytica* has an intense secretory activity and is considered a promising platform to produce a wide range of metabolites of industrial interest (Lopes et al. 2022). Citric acid is one of the most important organic acids produced by *Y. lipolytica*, with applications in the food and beverage industries as a flavoring and antioxidant agent, in the pharmaceutical industry as an excipient in formulations, and in the cosmetic, chemical, and detergent industries (Carsanba et al. 2019). The global production of citric acid in 2022 reached 2.59 million tons and citric acid production by fermentation is expected to grow continually at an annual growth rate of 5 % (Behera et al. 2021; Expert Market Research 2023). Other valuable compounds produced by *Y. lipolytica* include polyols like erythritol, mannitol, and arabitol, in response to osmotic stress (Egermeier et al. 2017; Vastaroucha et al. 2021). Polyols can be used as a sucrose substitute in food formulations due to their sweetening properties or utilized in the pharmaceutical industry as an excipient in formulations. The global market of polyols reached EUR 5.15 billion in 2019 and it is predicted an increase in polyols production at an annual growth rate of 6.1 % until 2027 (Diamantopoulou and Papanikolaou 2023).

Several works have proven *Yarrowia lipolytica* as a cell factory for the production of valuable compounds, however most of the practical applications of these compounds produced by *Y. lipolytica* remain in the exploratory stage. Few authors effectively produced biodiesel from lipids produced by yeast *Y. lipolytica* and lignocellulosic biomass (sugarcane bagasse) and waste cooking oil were substrates used to produce microbial lipids (Tsigie et al. 2012a; Katre et al. 2018; Vasaki et al. 2022). Therefore, the main aim of this work was to produce microbial lipids by *Y. lipolytica* NCYC 2904 from low-cost substrates, crude glycerol, and VFAs, to be used as feedstock for biodiesel production. Fed-batch and two-stage batch cultures (TSC) of *Y. lipolytica* were carried out in a stirred tank lab-scale bioreactor for lipids production. Microbial lipids produced in fed-batch cultures were extracted, followed by conversion into biodiesel via an alkaline catalytic transesterification. To assess the quality of biodiesel, a characterization by FTIR and an analysis of biodiesel properties in comparison to EU biodiesel standards were conducted. Citric acid and polyols production was also evaluated in all *Y. lipolytica* NCYC 2904 cultures.

7.2. Materials and Methods

7.2.1. Yeast strain and inoculum preparation

Yarrowia lipolytica NCYC 2904 cells were preserved in cryo-stocks at - 80 ºC and pre-inoculum was prepared as described in chapter 3.2.1.

7.2.2. Bioreactor experiments

All experiments were performed in a 2-L stirred tank bioreactor (BIOSTAT® B Plus, Sartorius) at 27 °C with a specific airflow rate of 1 vvm and an agitation rate of 400 rpm. The culture pH was measured with a pH electrode (InPro3100/225/Pt100 type, Metter Toledo) and kept at 6.0 ± 0.5 by the addition of NaOH 2 M or HCl 2 M. An optical probe (InPro6860i/12/220/nA, Metter Toledo) was used to measure the dissolved oxygen concentration in the culture medium.

Yarrowia lipolytica NCYC 2904 growth and metabolites production were evaluated in fed-batch and TSC cultures. Fed-batch experiments started with a batch culture with an initial working volume of 700 mL (about 50 % of the final volume) of medium containing 20 $g \cdot L^{-1}$ crude glycerol, 0.5 $g \cdot L^{-1}$ corn steep liquor, and 0.5 $g \cdot L^{-1}$ of ammonium sulfate (C/N mass ratio of 75). After 48 h of batch culture, a glycerol-concentrated medium (100 $g \cdot L^{-1}$ crude glycerol, 2.5 $g \cdot L^{-1}$ corn steep liquor, and 2.5 $g \cdot L^{-1}$ of ammonium sulfate) was fed to the bioreactor by a peristaltic pump using two constant feeding flow rates: (1) 4.96 $mL \cdot h^{-1}$, where the dilution rate ranged from 0.005 h^{-1} to 0.003 h^{-1} (FB-A); and (2) 3.10 $mL \cdot h^{-1}$, where the dilution rate ranged from 0.004 h^{-1} to 0.002 h^{-1} (FB-B). After 144 h (FB-A) or 202 h (FB-B) of the fed-batch phase, the culture mode was switched to the batch mode, which lasted for 72 h. In FB-B experiments, a pulse of 18 $g \cdot L^{-1}$ pure VFAs mixture (6 $g \cdot L^{-1}$ acetate, 6 $g \cdot L^{-1}$ propionate, and 6 $g \cdot L^{-1}$ butyrate) was added to the culture at the end of the fed-batch phase.

A TSC culture was also carried out in the bioreactor, filled with 1.3 L of culture media composed of 60 $g \cdot L^{-1}$ crude glycerol, 1.5 $g \cdot L^{-1}$ corn steep liquor, and 1.5 $g \cdot L^{-1}$ of ammonium sulfate (C/N mass ratio of 75) - first stage. The initial concentration of crude glycerol corresponds to the glycerol consumed by yeast culture in the FB-B experiments. After 192 h of cultivation, corresponding to the final of the exponential growth phase, a pulse of 18 $g \cdot L^{-1}$ pure VFAs mixture (6 $g \cdot L^{-1}$ acetate, 6 $g \cdot L^{-1}$ propionate, and 6 $g \cdot L^{-1}$ butyrate) was added to the medium - second stage.

Crude glycerol, provided by Prio Energy – Prio Biocombustíveis, SA, was composed (w/w) of 82 % glycerol, 12 % water, 4.7 % NaCl, 0.5 % organic matter (not glycerol) and less than 0.01 % methanol.

7.2.3. Analytical methods

Culture samples were taken at regular intervals for measurement of biomass, glycerol, VFAs, citric acid, and polyols concentration and analysis of lipids content and long-chain fatty acids composition. The analysis of samples was performed as described in the chapter 3.2.6. Citric acid and polyols were quantified by HPLC using the same method described in the chapter 3.2.6.

7.2.4. Biodiesel production and characterization

Microbial lipids were extracted from *Y. lipolytica* NCYC 2904 cells with an automatic Soxhlet extraction system (Soxtec™ 8000, FOSS, Denmark) using petroleum ether as solvent at 70 °C for 11 h. The lipids extracted were converted into biodiesel via an alkaline catalytic transesterification method using pure NaOH as a catalyst and pure methanol as a solvent. The transesterification reaction was performed at an oil:methanol molar ratio of 1:6, 1% (w/w) of catalyst, and the mixture was incubated at 65 °C for 4 h. After incubation, the mixture was centrifuged at 5000 rpm for 10 min and the upper layer containing biodiesel was separated. The yield of biodiesel was calculated according to Equation 7.1:

$$\text{Biodiesel yield (\%)} = \frac{\text{mass of biodiesel (g)}}{\text{mass of lipids (g)}} \times 100 \qquad \text{(Equation 7.1)}$$

Fourier Transform Infra-Red (FTIR) spectrometer (ALPHA II, Bruker, USA) was used to analyze the structure of extracted lipids of *Y. lipolytica* NCYC 2904 and biodiesel produced. The absorbance spectra were recorded in a wavenumber range of 4000 cm^{-1} to 400 cm^{-1} using an average of 64 scans per sample and with a spectral resolution of 4 cm^{-1}.

The physical and chemical properties of biodiesel (kinematic viscosity, density, degree of unsaturation, oxidation stability, cold filter plugging point, and cetane number) were estimated by the BiodieselAnalyzer© Ver. 2.2 software (available on http://brteam.org/analysis/) (Talebi et al. 2014) using the composition of lipids fatty acids.

7.2.5. Statistical analysis

One-way analysis of variance (ANOVA) was used for the statistical analysis of the data as described in chapter 3.2.8.

7.3. Results and Discussion

7.3.1. Microbial lipids production by *Y. lipolytica* NCYC 2904

Yarrowia lipolytica NCYC 2904 has a high potential for microbial lipids production (Pereira et al. 2022b; Pereira et al. 2023) and, consequently, is a promising precursor of great importance for biodiesel production. This work evaluated fed-batch and TSC cultures of *Y. lipolytica* NCYC 2904 for lipids production from crude glycerol and VFAs.

The *Y. lipolytica* cell mass variation with time was similar in all the culture modes tested (Figure 7.1). Although similar biomass concentration was attained, the biomass yield attained during the feeding phase on FB-A (0.33 $g \cdot g^{-1} \pm 0.01$ $g \cdot g^{-1}$) was higher than that observed in FB-B (0.24 $g \cdot g^{-1} \pm 0.01$ $g \cdot g^{-1}$) since more glycerol was consumed in the last case. Similarly to this, a biomass yield on glycerol of 0.289 $g \cdot g^{-1} \pm 0.001$ $g \cdot g^{-1}$ was obtained in the TSC experiments. At the end of the glycerol feeding in FB-A culture, approximately 15 $g \cdot L^{-1}$ of glycerol remained in the culture medium, which was further consumed by *Y. lipolytica* in the batch phase (Figure 7.1A). By contrast, in the fed-batch experiments with the lowest glycerol feeding (FB-B), yeast cells consumed glycerol at the same rate as it was added and a residual concentration of glycerol remained in the medium (Figure 7.1B). Crude glycerol took 192 h to be assimilated in TSC culture, 58 h earlier than that observed in FB-B cultures. Moreover, it is worth noticing that biomass yields reported herein are higher than others found in the literature with *Y. lipolytica* W29 (approximately 0.21 $g \cdot g^{-1}$) (Gajdoš et al. 2017) and A101 (0.21 $g \cdot g^{-1}$) (Dobrowolski et al. 2016) strains growing in glycerol. A biomass yield of 0.32 $g \cdot g^{-1}$ was obtained in *Y. lipolytica* W29 cultures using a mixture of VFAs or glucose and VFAs as substrates (Pereira et al. 2022a). In our previous works, despite the lower biomass concentration attained, a biomass yield (cell mass per substrate mass consumed) of 0.48 $g \cdot g^{-1}$ was obtained for *Y. lipolytica* NCYC 2904 cultivated in 40 $g \cdot L^{-1}$ crude glycerol under non-limiting oxygen conditions (Pereira et al. 2023), suggesting that the dissolved oxygen can be a limiting factor on biomass yield. Dissolved oxygen concentration remained close to zero during the feeding phases and the 1st phase of TSC. *Yarrowia lipolytica* cells cultivated under a lower amount of dissolved oxygen could have a negative effect on cell growth since *Y. lipolytica* is a strictly aerobic yeast (Magdouli et al. 2018; Pereira et al. 2021).

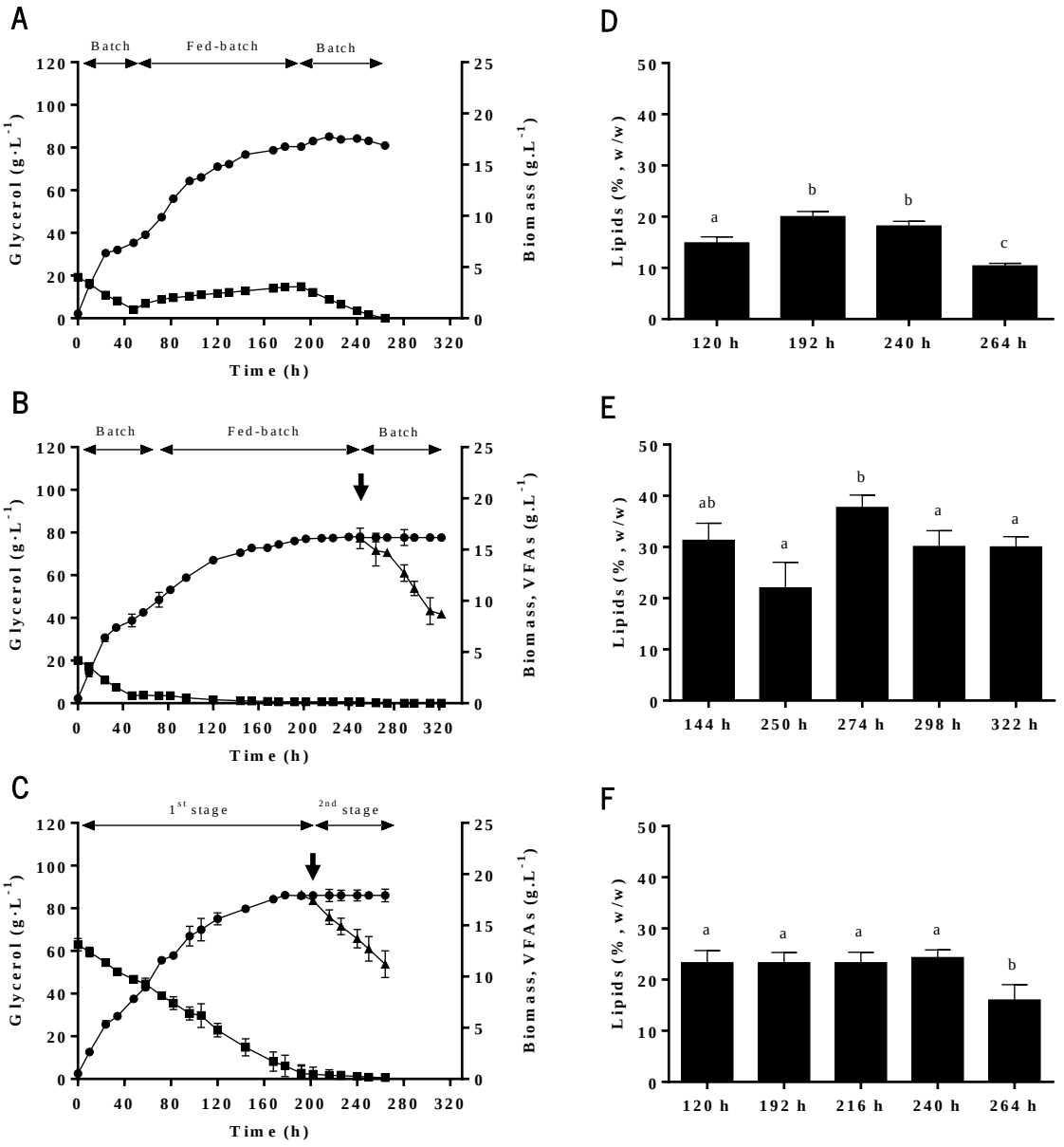


Figure 7.1 – Time course of biomass (●), glycerol (■), and total VFAs (▲) concentration obtained in *Y. lipolytica* NCYC 2904 cultures: FB-A (A), FB-B (B) and TSC culture (C). Microbial lipids content (%, w/w) of *Y. lipolytica* NCYC 2904 cells obtained in FB-A (D), FB-B (E) and TSC (F) cultures. The error bars represent the standard deviation of two independent replicates. Bars with the same letter are not statistically different ($p \geq 0.05$).

Regardless of the operation mode that precedes VFAs addition, no significant differences were observed in VFAs uptake rates (0.11 $g \cdot L^{-1} \cdot h^{-1} \pm 0.01$ $g \cdot L^{-1} \cdot h^{-1}$ in FB-B and 0.10 $g \cdot L^{-1} \cdot h^{-1} \pm 0.04$ $g \cdot L^{-1} \cdot h^{-1}$ in TSC). Unlike what was observed in our previous work (Pereira et al. 2022b), VFAs were not consumed by yeast after 72 h of cultivation (Figure 7.1B and 7.1C). The total VFAs uptake rates obtained in this work are lower

than those obtained in TSC (growth phase in glucose followed by 18 $g \cdot L^{-1}$ VFAs mixture) (Pereira et al. 2022b). Moreover, Pereira et al. (2022b) found that low dissolved oxygen concentration in the culture medium had a negative effect on VFAs consumption by yeast, which could explain the low VFAs consumption observed in the present work. Despite the carbon sources being consumed, no cellular growth was observed in the batch phase (fed-batch experiments) and in 2nd stage of TSC (Figure 7.1A, 7.1B, and 7.1C). This could indicate that carbon sources were channeled toward cell maintenance or metabolites production instead biomass production. Similar behavior was described in previous works, in which a decrease in cell proliferation was observed after the addition of VFAs, being the carbon sources assimilated by yeast for lipids synthesis (Pereira et al. 2022b; Pereira et al. 2023).

Although no differences were observed in *Y. lipolytica* growth, microbial lipids production had different profiles, depending on the culture mode (Figure 7.1D, 7.1E and 7.1F). In the case of FB-A culture, lipids content increased and a 1.4-fold improvement in lipids content was achieved after 192 h compared to 120 h. The amount of microbail lipids at the end of the experiment was half that obtained at the end of the feeding phase (192 h) (Figure 7.1D). In FB-B culture, no statistical differences were observed in microbial lipids production during the feeding phase. A 1.7-fold improvement in lipids content was obtained after 24 h VFAs addition (274 h of cultivation). The VFAs addition without an extra nitrogen source led to an increase in the C/N ratio, which could favor microbial lipids production. Although lipids content attained at the end of the experiments was 1.3 times lower than attained at 274 h, it is worth noticing that no statistical differences were observed at the end of the feeding phase (250 h) and the end of FB-B culture (Figure 7.1E). According to Poontawee and Limtong (2020), microbial lipids synthesis was enhanced at the lowest feeding rate. Although no differences were observed in lipids content at the end of the feeding phase, a 2-fold improvement in maximum lipids productivity was obtained by decreasing the substrate feeding rate from 0.5 $g \cdot h^{-1} \cdot L^{-1}$ (FB-A) to 0.4 $g \cdot h^{-1} \cdot L^{-1}$ (FB-B). In TSC culture, no statistical differences were observed in lipids content until 240 h of cultivation, and mobilization of lipids content was observed after 72 h of VFAs addition. A 1.5- fold decrease in lipids content was observed at the end of the experiments (Figure 7.1F). Microbial lipids accumulation by *Y. lipolytica* obtained at the end of 1st stage (192 h) was similar to that reached at the end of the feeding phase in FB-B culture, suggesting that microbial lipids production was modeled by glycerol concentration and not cultivation mode since the same amount of glycerol was consumed by yeast in both experiments. While no differences in maximum lipids productivity were observed in TSC and FB-B cultures, higher productivity was attained in TSC cultures relative to FB-A cultures. A mobilization of synthesized microbial lipids was observed in the batch phase of fed-batch experiments and 2nd stage of TCS culture, even though all

glycerol was consumed by yeast (FB-A) and a significant amount of VFAs remained unconsumed in the medium (FB-B and TSC) (Figure 7.1). Besides producing microbial lipids, *Y. lipolytica* cultivated in nitrogen-limited conditions is also triggered towards the production of low-molecular-weight metabolites (citric acid or polyols) (Papanikolaou et al. 2020). Microbial lipids mobilization and secretion of low-molecular-weight metabolites were observed for *Y. lipolytica* ACA YC 5030 (Papanikolaou et al. 2017), ACA-DC 50109 (Bellou et al. 2016), and FMCC Y_{75} (Filippousi et al. 2019) on glycerol-based media under nitrogen-limited conditions. The maximum lipids content of 38 % (w/w) achieved in FB-B cultures was 1.9- and 1.6- fold higher than that attained in FB-A and TSC cultures, respectively. Moreover, the maximum lipids concentration obtained in FB-B cultures (6.1 $g \cdot L^{-1}$) was 1.8- and 1.4-fold higher than that in FB-A and TSC cultures, respectively. To our best knowledge, no works have tested similar strategies for microbial lipids production from crude glycerol and VFAs, however, the maximum value of lipids production in fed-batch cultures tested herein is comparable to or even higher than that attained in *Y. lipolytica* fed-batch cultures with a pulse of glycerol (Kumar et al. 2020; Papanikolaou et al. 2020). Furthermore, similar lipids content was reported in *Y. lipolytica* NCYC 2904 batch cultures on crude glycerol (Pereira et al. 2023). Yet, the highest lipids concentration was obtained in this study, since cellular density was increased in fed-batch cultures.

7.3.2. Other added-value metabolites production by *Y. lipolytica* NCYC 2904

Simultaneously with microbial lipids, considerable amounts of citric acid and polyols (mannitol and arabitol) were produced by *Y. lipolytica* (Figure 7.2). Glycerol assimilated by yeast cells is first phosphorylated to glycerol-3-phosphate by glycerol kinase and then oxidized to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase. Dihydroxyacetone phosphate is converted into glycerol-3-glyceraldehyde, which can be used in gluconeogenesis (production of hexoses and polyols) or enters in glycolysis (production of lipids and organic acids). In glycolysis, glycerol-3-glyceraldehyde is converted into pyruvic acid and then acetyl-CoA, a precursor of a variety of metabolites. Under low nitrogen concentration conditions, lipids and/or citric acid are produced (Carsanba et al. 2018; Lopes et al. 2022). The simultaneous production of lipids and citric acid by *Y. lipolytica* from glycerol was already reported in fed-batch cultures with a pulse of glycerol (Kumar et al. 2020). Citric acid production was 2 times higher in fed-batch experiments than in TSC cultures. Moreover, acid citric production at the end of glycerol feeding in FB-B culture (250 h) was 1.7-fold higher than that attained in FB-A culture (192 h) (Figure 7.2A). Karamerou et al. (2017) applied different glycerol feeding rates for lipids production by *Rhodotorula glutinis*, demonstrating that increasing feeding rate resulted in low citric acid production.

Yarrowia lipolytica cells still produced citric acid from glycerol in the batch phase of FB-A culture simultaneously with lipid turnover (Figure 7.1D and 7.2A). In turn, a continuous citric acid increase was observed in FB-B and TSC cultures until VFAs addition, the moment that yeast cells stop producing citric acid. In TSC, the maximum acid citric production occurred at 192 h of cultivation (corresponding to the time that all glycerol was consumed), remaining in this value after VFAs addition (Figure 7.2C).A similar behavior occurred in FB-B cultures where the maximum citric acid production was observed at the end of the feeding phase (250 h) and no further citric acid production was observed after VFAs addition (Figure 7.2B).

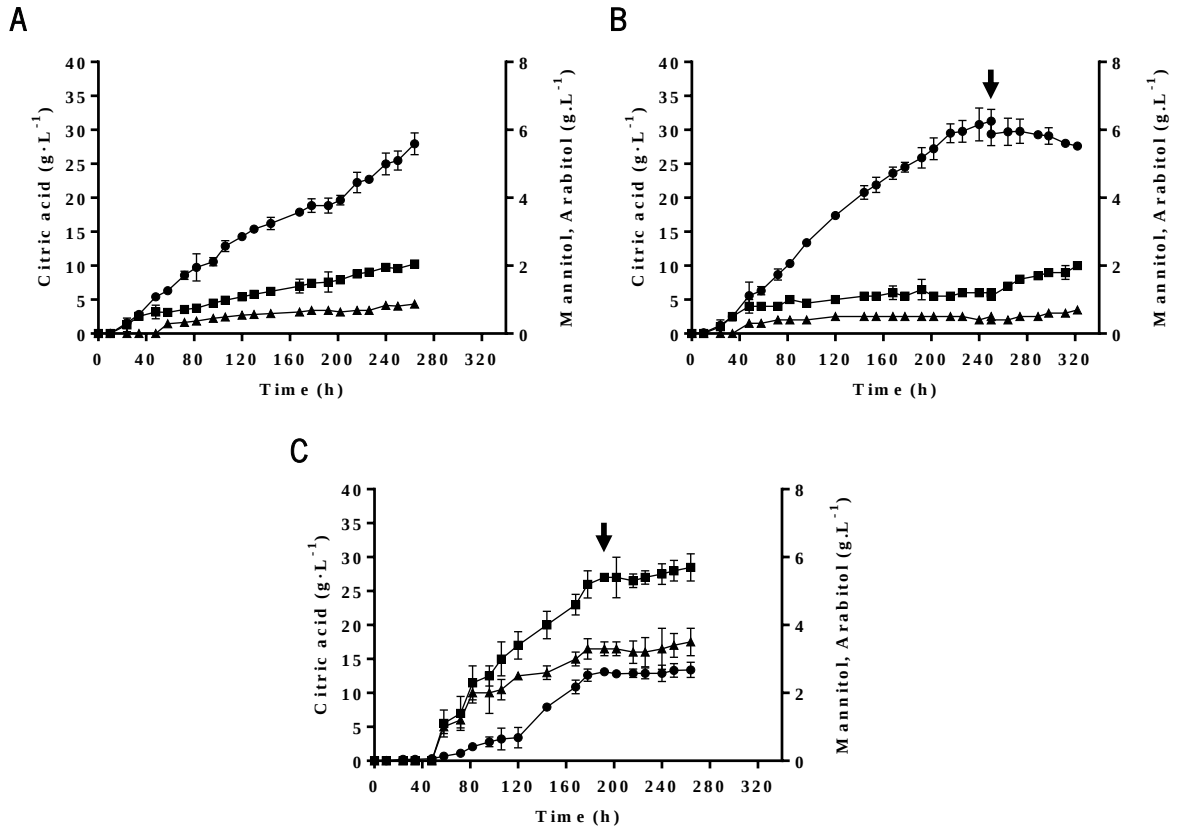


Figure 7.2 - Profiles of citric acid (●), mannitol (■), and arabitol (▲) concentration obtained in *Y. lipolytica* cultures: FB-A (A), FB-B (B), and TSC (C) cultures. The error bars represent the standard deviation of two independent replicates.

Yarrowia lipolytica also produced polyols (mainly mannitol and arabitol) from crude glycerol, particularly in TSC experiments (Figure 7.2). Though some studies reported the synthesis of erythritol from crude glycerol (Filippousi et al. 2019; Vastaroucha et al. 2021), no erythritol production was found in this work. Contrarily to citric acid, the highest amount of mannitol and arabitol produced by *Y. lipolytica* was attained

in TSC cultures. TSC cultures reached 2.8- fold higher mannitol concentration than in fed-batch cultures. Additionally, arabitol concentration in TSC was 4- and 5-fold higher than that obtained in FB-A and FB-B cultures, respectively. The higher initial concentration of glycerol in the culture medium can increase osmotic pressure, which may explain the highest polyols production in TSC cultures. Mannitol was the main polyol in all cultures, whereas arabitol was produced in the lowest amounts. Although polyols production occurs mainly when cultures are carried out at low pH (Egermeier et al. 2017), *Y. lipolytica* was able to produce polyols in the present work conditions. Similarly, it has been demonstrated that other *Y. lipolytica* strains cultivated under nitrogen limitation can produce polyols at medium pH values of 6 (Papanikolaou et al. 2017; Papanikolaou et al. 2020). Furthermore, mannitol was produced from VFAs, since a 1.7-fold increase in mannitol concentration was observed after VFAs addition in FB.B cultures (Figure 7.2B). In TSC cultures, no polyols production was observed after VFAs addition, probably due to (a) metabolites other than those we measured being produced; (b) high substrate consumption for cellular maintenance; (c) the operation time, cell death is higher than cell proliferation and reproduction.

The current study shows the relevance of *Y. lipolytica* as a cell factory to convert crude glycerol and VFAs into bioproducts of industrial interest. *Yarrowia lipolytica* has great potential for the production of a high amount of citric acid (which can be used in the food and beverage industry) along with lipids-rich biomass (used as feedstock to biodiesel production) and polyols (used as a sweetener), what reduces the production costs of each bioproduct, making these bioprocesses integrated within a biorefinery framework. Moreover, depending on the needs of the market, different routes may be selected by changing operating modes, such as fed-batch operation mode to produce citric acid or batch cultures to produce mannitol.

7.3.3. Biodiesel production

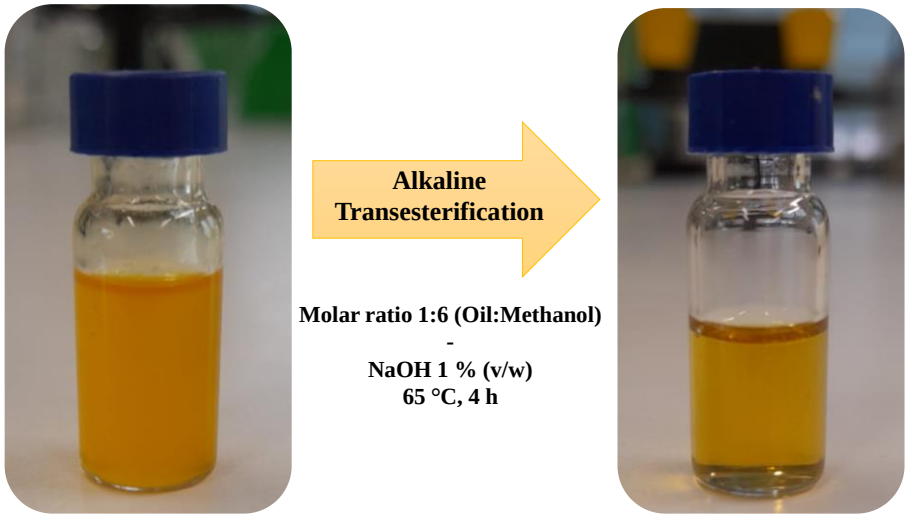
Microbial lipids produced at the end of *Y. lipolytica* cultures were mainly composed of unsaturated fatty acid (> 84 %), whose fatty acid composition was similar to vegetable oils, traditionally used as raw feedstock for biodiesel production. Oleic acid (C18:1) was the main fatty acid produced, followed by palmitoleic (C16:1). Linoleic (C18:2), palmitic (C16:0), and stearic (C18:0) acids were also synthesized in low quantities (Table 7.1). As in other works using VFAs, heptadecanoic acid (C17:1) was synthesized owing to the addition of propionate into the culture medium (Pereira et al. 2022b). Moreover, a slight difference in the relative percentage of each fatty acid was observed between FB-B and TSC cultures. While in FB-B cultures *Y. lipolytica* produced more linoleic acid, TSC cultures led to a decrease of linoleic

and oleic acids and an increase in palmitic acid contents. Similar fatty acid profiles were found by other *Y. lipolytica* strains grown in pure or crude glycerol (Papanikolaou et al. 2017; Papanikolaou et al. 2020), VFAs (Llamas et al. 2020a; Pereira et al. 2022a) or hydrophobic substrates (Lopes et al. 2018; Fabiszewska et al. 2021).

Table 7.1- Fatty acids composition of microbial lipids produced by *Y. lipolytica* NCYC 2904 under different cultivations mode in stirred tank bioreactor. Data are average ± standard deviation of two independent replicates. Values followed by the same letter in each column do not present statistically significant differences ($p \geq 0.05$).

Y. lipolytica culture	Relative fatty acid content (%)					
	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2
FB-A	7.6 ± 0.1 ^{a}	18.47 ± 0.01 ^{a}	-	3.8 ± 0.1 ^{ab}	62.6 ± 0.2 ^{a}	7.51 ± 0.02 ^{a}
FB-B	6.4 ± 0.5 ^{a}	15.8 ± 0.8 ^{a}	2.3 ± 0.3 ^{a}	3.3 ± 0.5 ^{a}	60.9 ± 0.5 ^{a}	11.3 ± 0.2 ^{b}
TSC	10 ± 1 ^{b}	17 ± 2 ^{a}	2.1 ± 0.2 ^{a}	6 ± 1 ^{b}	58 ± 2 ^{b}	7.4 ± 0.8 ^{a}

Yarrowia lipolytica lipids produced in FB-B cultures (the condition where a higher amount of lipids was obtained) were extracted and then submitted to an alkaline catalytic transesterification process resulting in 95 % biodiesel yield (Figure 7.3).



Bio-oil extracted from *Y. lipolytica* cells

Biodiesel

Figure 7.3 – Microbial lipids extracted from *Y. lipolytica* NCYC 2904 cells and biodiesel produced via alkaline catalytic transesterification of lipids.

The structural characterization of *Y. lipolytica* lipids extracted from cells growing in the FB-B culture and biodiesel produced from microbial lipids was visualized using FTIR spectra (Figure 7.4). Additionally, FTIR analysis of vegetable oil (commercial oil used for food purposes) and biodiesel produced from vegetable oil was carried out for comparison purposes. FTIR spectra of *Y. lipolytica* lipids and vegetable oil showed similar characteristics although with small differences in spectral bands and absorbance (Figure 7.4A and 7.4B). In *Y. lipolytica* lipids, the different lipid functional groups were represented by the strong peaks at 1457.5 cm^{-1} (C–H bend of methyl group), 1744.1 cm^{-1} (stretching vibration of ester carbonyl bond C=O), 2855.3 cm^{-1} and 2925.4 cm^{-1} (asymmetric and symmetric stretching vibrations for C–H saturated carbon chains) (Radha et al. 2020). It should be noticed that the FTIR spectrum of vegetable oil showed also the presence of bands at 1459.6 cm^{-1}, 1744.1 cm^{-1}, 2853.2 cm^{-1}, and 2923.3 cm^{-1}. Similar spectral peaks for microbial lipids extracted from *Y. lipolytica* as a potential feedstock for biodiesel production were also reported in other studies performed by Mitrea et al. (2022), Radha et al. (2020) and Vasaki et al. (2022).

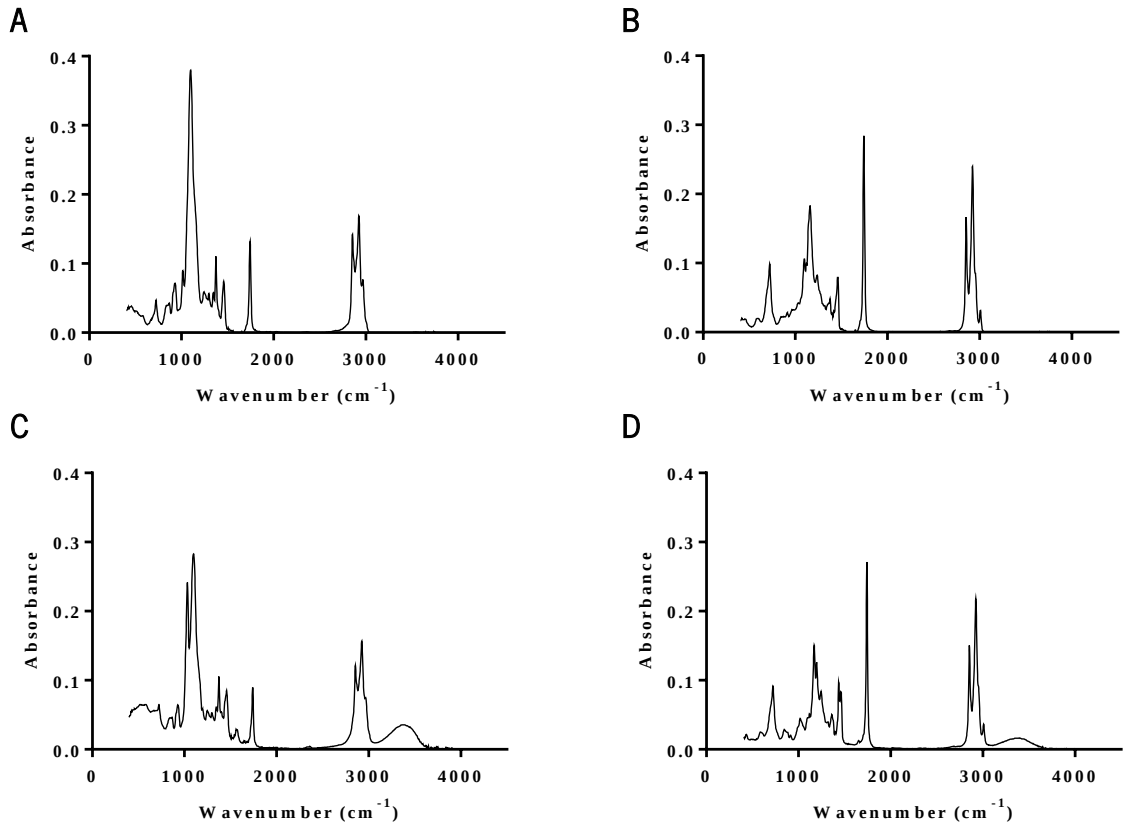


Figure 7.4 - FTIR spectrum of microbials lipids derived from *Y. lipolytica* cells in FB-B culture (A), and FTIR spectrum of vegetable oil (B). FTIR spectrum of biodiesel obtained by the alkaline catalytic transesterification of *Y. lipolytica* lipids (C) and vegetable oil (D).

The FTIR spectrum of methyl esters in *Y. lipolytica* lipids has shown peaks at 2855.3 cm^{-1} and 2927.3 cm^{-1}, corresponding to sp^2 C–H and sp^3 C–H stretching, respectively. At 1740 cm^{-1}, the spectral peak represents the C=O stretch in the ester group as observed in the FTIR spectrum of *Y. lipolytica* lipids. The main change from the ester group to methyl ester occurred in the region of 400 cm^{-1} - 1500 cm^{-1} (Figure 7.4C). The broad and strong peak at 1096.8 cm^{-1} in the FTIR spectrum of lipids (Figure 7.4A) was split into two peaks at 1030.8 cm^{-1} and 1098.8 cm^{-1} in FTIR spectra of biodiesel (Figure 7.4C), which represents C–C asymmetric vibrations in aliphatic esters. Another middling absorbance signal at 3007.9 cm^{-1} was also found. These results are in accordance with other studies that analyzed the functional groups corresponding to methyl groups in biodiesel samples for confirming the occurrence of fatty acid methyl esters (Munir et al. 2019; Bibi et al. 2022). Additionally, the spectral analysis of methyl esters derived from the transesterified vegetable oil exhibited similar characteristics to those observed in biodiesel obtained by transesterification of *Y. lipolytica* lipids.

The potential of *Y. lipolytica* biodiesel to replace conventional fuel was evaluated to ensure that the biodiesel produced meets the criteria set up by international biodiesel standards EN 14214. The fatty acid composition of *Y. lipolytica* lipids was used to estimate several properties using the BiodieselAnalyzer© software. Table 7.2 shows the main parameters used to assess the biodiesel quality and compared with biodiesel produced from soybean oil (one of the most vegetable oils used as feedstock in biodiesel production) and international biodiesel standard.

Table 7.2 - Properties of biodiesel obtained from *Y. lipolytica* NCYC 2904 lipids and soybean oil estimated by BiodieselAnalyzer© software and the EU biodiesel standard EN 14214.

Properties	Biodiesel from *Y. lipolytica* NCYC 2904 lipids	Biodiesel from soybean oil (Yaşar 2020)	EU biodiesel standard EN 14214
Density ($kg \cdot m^{-3}$)	856	882	860 – 900
Kinematic viscosity ($mm^2 \cdot s^{-1}$)	3.71	4.37	3.5 – 5.0
Cetane number	53.31	51	47 min
Oxidation Stability (h)	13.03	1.5	6 h min
Cold filter plugging point (°C)	- 9.28	- 6	NR*

NR – not reported; NR*– Cold filter plugging point limits depend on geography and time of the year; min – minimum; max – maximum; h – hours.

The properties of biodiesel produced from *Y. lipolytica* lipids agree with the specifications of EU biodiesel standard EN 14214 and demonstrate the high quality of the biodiesel produced (Table 7.2). Furthermore, the biodiesel properties (density, kinematic viscosity, and cetane number) obtained from *Y. lipolytica* NCYC 2904 lipids showed high similarity with those of biodiesel produced from soybean oil. However, the value estimated of oxidation stability (property to evaluate storage behavior) is above the EU standards minimum thresholds and is higher than that obtained in biodiesel from vegetable oil. The cold filter plugging point estimated in this work is slightly lower than that obtained in biodiesel produced from soybean oil and lower than those obtained with *Y. lipolytica* lipids produced in other studies (Pereira et al. 2022b; Pereira et al. 2022a). Although there is no specific recommendation in the EU standards, the lower the cold filter plugging point value, the better the cold flow properties of the biodiesel. Hence, *Y. lipolytica* lipids synthesized from crude glycerol and VFAs in FB-B cultures have the potential to be used as feedstock for the production of high-quality biodiesel.

7.4. Conclusions

The results obtained in this work are quite promising since promote a model of circular economy, closing the loop from crude glycerol to biodiesel. Crude glycerol, the main by-product generated during biodiesel production, and VFAs were used as renewable and low-cost feedstocks for microbial lipids production and other metabolites of industrial interest (citric acid and polyols) by the oleaginous yeast *Y. lipolytica* NCYC 2904. A fed-batch culture with crude glycerol feed at the lowest feeding rate, followed by the addition of VFAs mixture, resulted in a high amount of microbial lipids, rich in oleic acid, which were used to produce biodiesel with a 95 % conversion yield. The estimated biodiesel properties demonstrated that biodiesel obtained by transesterification of *Y. lipolytica* lipids can be used as high-quality biofuel.

CHAPTER 8

General conclusions and final remarks

8.1. General conclusions

Yarrowia lipolytica has been attracting scientific and industrial attention as a biorefinery platform for sustainable microbial lipids production. Using low-cost feedstocks as carbon sources for microbial growth and lipids production is promising for implementing sustainable bioprocesses under the scope of the circular economy concept. Therefore, the development of reliable and sustainable processes to obtain microbial lipids from low-cost feedstocks is needed. In particular, this thesis focused on the development of bioprocess based on VFAs generated from food waste to obtain raw-material for biodiesel production using the yeast *Y. lipolytica*. The feasibility of two strains of *Y. lipolytica* (W29 and NCYC 2904) to produce microbial lipids under different operation conditions and operation modes was studied in order to maximize microbial lipids production.

Yarrowia lipolytica W29 and NCYC 2904 demonstrated to be suitable candidates for lipids production from VFAs. The addition of co-substrates (glucose or glycerol), oxygen and operation mode proved to be factors affecting the bioconversion of VFAs (acetate, propionate and butyrate) by both strains for growth and microbial lipids production. Moreover, a two-stage batch culture revealed to be a high potential strategy to boost lipids biosynthesis from VFAs.

A set of different two-stage batch culture approaches were conducted to assess microbial lipids production from high-content VFAs media. A two-stage batch culture - growth phase in glucose, followed by a lipogenic phase in 18 $g \cdot L^{-1}$ VFAs was an efficient strategy to improve lipids production. For *Y. lipolytica* NCYC 2904, the effect of dissolved oxygen concentration during the lipogenic phase on lipid production was assessed in a lab-scale stirred bioreactor, revealing that dissolved oxygen concentration between 40% and 50% of saturation during the lipogenic phase led to an increase of lipids production. The fatty acids profile produced by both strains, mainly consisting of oleic acid, was similar to that of vegetable oils commonly used in the biodiesel industry. Remarkably, odd-chain fatty acids (heptadecenoic and margaric acids) were produced by both *Y. lipolytica* strains. Moreover, the estimated properties of biodiesel produced by microbial lipids are within a range of international biodiesel standards demonstrating their potential as raw-material for biodiesel production.

The ability of *Y. lipolytica* W29 and NCYC 2904 to grow in VFAs-rich fermented medium obtained in acidogenic fermentation of food waste was demonstrated, validating the possibility of using this VFAs-rich fermented medium as a low-cost carbon source by the *Y. lipolytica* strains used in this study. With a remarkable potential for biomass production by NCYC 2904 strain, microbial lipids production by *Y. lipolytica* NCYC 2904 in batch and two-stage batch cultures was assessed in a lab-scale stirred bioreactor.

Once again, two-stage batch culture demonstrated to be a reliable strategy of lipids production. The highest amounts of lipids (11 $g \cdot L^{-1}$) were produced in a two-stage batch culture using a VFAs-rich fermented medium, followed by the addition of two pulses of crude glycerol, demonstrating the suitability of using VFAs-rich fermented medium from acidogenic fermentation and crude glycerol as low-cost substrates for lipids production.

Given that *Y. lipolytica* NCYC 2904 was found to be the most promising strain in terms of biomass and lipids production, the potential of *Y. lipolytica* NCYC 2904 as a cell factory in the production of feedstock for biodiesel from low-cost substrates was investigated in fed-batch and two-stage batch cultures. Although bioprocess performance would require further studies of optimization, fed-batch cultures proved to be an effective strategy for lipids production simultaneous with other bioproducts of industrial interest (citric acid and polyols). Moreover, *Y. lipolytica* lipids was effectively converted into biodiesel, whose main properties are within the international biodiesel standards, proving the high-quality biodiesel production from microbial lipids.

8.2. Future perspectives

Overall, the research work presented in this thesis has demonstrated the successful utilization of alternative carbon sources derived from organic wastes, such as VFAs generated during anaerobic fermentation and crude glycerol, to enhance the viability of the microbial lipids production bioprocess and following application in biodiesel production. Nevertheless, further studies could be performed to attain efficient microbial lipids production from low-cost feedstocks, such as:

- Further optimization of parameters of culture medium, such as, nitrogen and phosphorous sources and concentration, and other supplementations like mineral salts that could increase microbial lipids production.
- Further optimization of different C/N ratio in the medium in the two-stage batch cultures to maximize the lipids production.
- Apply similar two-stage batch culture developed during this thesis for VFAs-rich fermented medium produced by acidogenesis from different organic waste sources, since the concentration and composition of VFAs vary according the substrate and fermentation condition of anaerobic fermentation.
- *In-situ* transesterification for the direct conversion of yeast biomass to biodiesel could be tested in future works, since the combination of lipids extraction and transesterification in a single step reduces the overall cost of this bioprocess.

- Further studies focusing on the microbial lipids production from other low-cost organic wastes or hydrolysates from agricultural and forest residues could be performed in order to broaden the biorefinery approach.

CHAPTER 9

References

Agnihotri S, Yin D-M, Mahboubi A, Sapmaz T, Varjani S, Qiao W, Koseoglu-Imer DY, Taherzadeh MJ (2022) A Glimpse of the world of volatile fatty acids production and application: a review. Bioengineered 13:1249–1275. https://doi.org/10.1080/21655979.2021.1996044

Alonso FOM, Oliveira EBL, Dellamora-Ortiz GM, Pereira-Meirelles FV (2005) Improvement of lipase production at different stirring speeds and oxygen levels. Brazilian Journal of Chemical Engineering 22:9–18. https://doi.org/10.1590/S0104-66322005000100002

Annamalai N, Sivakumar N, Oleskowicz-popiel P (2018) Enhanced production of microbial lipids from waste office paper by the oleaginous yeast *Cryptococcus curvatus*. Fuel 217:420–426. https://doi.org/10.1016/j.fuel.2017.12.108

Asadi P, Rad HA, Qaderi F (2020) Lipid and biodiesel production by cultivation isolated strain *Chlorella sorokiniana* pa.91 and *Chlorella vulgaris* in dairy wastewater treatment plant effluents. J Environ Health Sci Eng 18:573–585. https://doi.org/10.1007/s40201-020-00483-y

Atasoy M, Owusu-Agyeman I, Plaza E, Cetecioglu Z (2018) Bio-based volatile fatty acid production and recovery from waste streams: Current status and future challenges. Bioresour Technol 268:773–786. https://doi.org/10.1016/j.biortech.2018.07.042

Bao W, Li Z, Wang X, Gao R, Zhou X, Cheng S, Men Y, Zheng L (2021) Approaches to improve the lipid synthesis of oleaginous yeast *Yarrowia lipolytica*: A review. Renewable and Sustainable Energy Reviews 149:111386. https://doi.org/10.1016/j.rser.2021.111386

Behera BC, Mishra R, Mohapatra S (2021) Microbial citric acid: Production, properties, application, and future perspectives. Food Front 2:62–76. https://doi.org/10.1002/fft2.66

Bellou S, Makri A, Triantaphyllidou I-E, Papanikolaou S, Aggelis G (2014) Morphological and metabolic shifts of *Yarrowia lipolytica* induced by alteration of the dissolved oxygen concentration in the growth environment. Microbiology (N Y) 160:807–817. https://doi.org/10.1099/mic.0.074302-0

Bellou S, Triantaphyllidou I-E, Mizerakis P, Aggelis G (2016) High lipid accumulation in *Yarrowia lipolytica* cultivated under double limitation of nitrogen and magnesium. J Biotechnol 234:116–126. https://doi.org/10.1016/j.jbiotec.2016.08.001

Beopoulos A, Cescut J, Haddouche R, Uribelarrea J-L, Molina-Jouve C, Nicaud J-M (2009) *Yarrowia lipolytica* as a model for bio-oil production. Prog Lipid Res 48:375–387. https://doi.org/10.1016/j.plipres.2009.08.005

Bhatia SK, Gurav R, Choi T-R, Jung H-R, Yang S-Y, Song H-S, Kim Y-G, Yoon J-J, Yang Y-H (2019) Effect of synthetic and food waste-derived volatile fatty acids on lipid accumulation in *Rhodococcus* sp. YHY01 and the properties of produced biodiesel. Energy Convers Manag 192:385–395. https://doi.org/10.1016/j.enconman.2019.03.081

Bialy H El, Gomaa OM, Azab KS (2011) Conversion of oil waste to valuable fatty acids using oleaginous yeast. World J Microbiol Biotechnol 27:2791–2798. https://doi.org/10.1007/s11274-011-0755-x

Bibi F, Ishtiaq Ali M, Ahmad M, Bokhari A, Shiong Khoo K, Zafar M, Asif S, Mubashir M, Han N, Loke Show P (2022) Production of lipids biosynthesis from *Tetradesmus nygaardii* microalgae as a feedstock for biodiesel production. Fuel 326:124985. https://doi.org/10.1016/j.fuel.2022.124985

Bórawski P, Bełdycka-Bórawska A, Szymańska EJ, Jankowski KJ, Dubis B, Dunn JW (2019) Development of renewable energy sources market and biofuels in The European Union. J Clean Prod 228:467–484. https://doi.org/10.1016/j.jclepro.2019.04.242

Brabender M, Hussain MS, Rodriguez G, Blenner MA (2018) Urea and urine are a viable and cost-effective nitrogen source for *Yarrowia lipolytica* biomass and lipid accumulation. Appl Microbiol Biotechnol 102:2313–2322. https://doi.org/10.1007/s00253-018-8769-z

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Braga A, Freitas B, Cordeiro A, Belo I (2021) Valorization of crude glycerol as carbon source for the bioconversion of L-phenylamine to 2-phenylethanol by *Yarrowia* species. Journal of Chemical Technology & Biotechnology 96:2940–2949. https://doi.org/10.1002/jctb.6849

Braga A, Mesquita DP, Amaral AL, Ferreira EC, Belo I (2015) Aroma production by *Yarrowia lipolytica* in airlift and stirred tank bioreactors: Differences in yeast metabolism and morphology. Biochem Eng J 93:55–62. https://doi.org/10.1016/j.bej.2014.09.006

Brar KK, Sarma AK, Aslam M, Polikarpov I, Chadha BS (2017) Potential of oleaginous yeast *Trichosporon* sp., for conversion of sugarcane bagasse hydrolysate into biodiesel. Bioresour Technol 242:161–168. https://doi.org/10.1016/j.biortech.2017.03.155

Cao L, Yin M, Shi T-Q, Lin L, Ledesma-Amaro R, Ji X-J (2022) Engineering *Yarrowia lipolytica* to produce nutritional fatty acids: Current status and future perspectives. Synth Syst Biotechnol 7:1024–1033. https://doi.org/10.1016/j.synbio.2022.06.002

Caporusso A, Capece A, de Bari I (2021) Oleaginous yeasts as cell factories for the sustainable production of microbial lipids by the valorization of agri-food wastes. Fermentation 7:1-33. https://doi.org/10.3390/fermentation7020050

Caporusso A, De Bari I, Liuzzi F, Albergo R, Valerio V, Viola E, Pietrafesa R, Siesto G, Capece A (2023) Optimized conversion of wheat straw into single cell oils by *Yarrowia lipolytica* and *Lipomyces tetrasporus* and synthesis of advanced biofuels. Renew Energy 202:184–195. https://doi.org/10.1016/j.renene.2022.11.059

Carmona-Cabello M, García IL, Papadaki A, Tsouko E, Koutinas A, Dorado MP (2021) Biodiesel production using microbial lipids derived from food waste discarded by catering services. Bioresour Technol 323:124597. https://doi.org/10.1016/j.biortech.2020.124597

Carsanba E, Papanikolaou S, Erten H (2018) Production of oils and fats by oleaginous microorganisms with an emphasis given to the potential of the nonconventional yeast *Yarrowia lipolytica*. Crit Rev Biotechnol 38:1230–1243. https://doi.org/10.1080/07388551.2018.1472065

Carsanba E, Papanikolaou S, Fickers P, Erten H (2020) Lipids by *Yarrowia lipolytica* strains cultivated on glucose in batch Cultures. Microorganisms 8:1054. https://doi.org/10.3390/microorganisms8071054

Carsanba E, Papanikolaou S, Fickers P, Erten H (2019) Screening various *Yarrowia lipolytica* strains for citric acid production. Yeast 36:319–327. https://doi.org/10.1002/yea.3389

Casal M, Paiva S, Queirós O, Soares-Silva I (2008) Transport of carboxylic acids in yeasts. FEMS Microbiol Rev 32:974–994. https://doi.org/10.1111/j.1574-6976.2008.00128.x

Chatterjee S, Mohan SV (2020) Yeast fermentation towards biodiesel: Maximizing resource recovery by integrating with biohydrogen production in biorefinery framework. Biomass Bioenergy 142:105747. https://doi.org/10.1016/j.biombioe.2020.105747

Chew KR, Leong HY, Khoo KS, Vo D-VN, Anjum H, Chang C-K, Show PL (2021) Effects of anaerobic digestion of food waste on biogas production and environmental impacts: a review. Environ Chem Lett 19:2921–2939. https://doi.org/10.1007/s10311-021-01220-z

Christophe G, Deo JL, Kumar V, Nouaille R, Fontanille P, Larroche C (2012) Production of oils from acetic acid by the oleaginous yeast *Cryptococcus curvatus*. Appl Biochem Biotechnol 167:1270–1279. https://doi.org/10.1007/s12010-011-9507-5

Coelho MA, Amaral P, Belo I (2010) *Yarro*wia *lipolytica*: an industrial workhorse. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, 2, 930-940.

Dahiya S, Lingam Y, Venkata Mohan S (2023) Understanding acidogenesis towards green hydrogen and volatile fatty acid production – Critical analysis and circular economy perspective. Chemical Engineering Journal 464:141550. https://doi.org/10.1016/j.cej.2023.141550

Dharmaraja J, Nguyen DD, Shobana S, Saratale GD, Arvindnarayan S, Atabani AE, Chang SW, Kumar G (2019) Engine performance, emission and bio characteristics of rice bran oil derived biodiesel blends. Fuel 239:153–161. https://doi.org/10.1016/j.fuel.2018.10.123

Diamantopoulou P, Filippousi R, Antoniou D, Varfi E, Xenopoulos E, Sarris D, Papanikolaou S (2020) Production of added-value microbial metabolites during growth of yeast strains on media composed of biodiesel-derived crude glycerol and glycerol/xylose blends. FEMS Microbiol Lett 367:1-13. https://doi.org/10.1093/femsle/fnaa063

Diamantopoulou P, Papanikolaou S (2023) Biotechnological production of sugar-alcohols: Focus on *Yarrowia lipolytica* and edible/medicinal mushrooms. Process Biochemistry 124:113–131. https://doi.org/10.1016/j.procbio.2022.11.008

Dobrowolski A, Mituła P, Rymowicz W, Mirończuk AM (2016) Efficient conversion of crude glycerol from various industrial wastes into single cell oil by yeast *Yarrowia lipolytica*. Bioresour Technol 207:237–243. https://doi.org/10.1016/j.biortech.2016.02.039

Duarte MS, Oliveira J V, Pereira C, Carvalho M, Mesquita DP, Alves MM (2021) Volatile Fatty Acids (VFA) production from wastewaters with high salinity - Influence of pH, salinity and reactor configuration. Fermentation 7:303. https://doi.org/10.3390/fermentation7040303

Dulermo T, Nicaud J-M (2011) Involvement of the G3P shuttle and β-oxidation pathway in the control of TAG synthesis and lipid accumulation in *Yarrowia lipolytica*. Metab Eng 13:482–491. https://doi.org/10.1016/j.ymben.2011.05.002

Egermeier M, Russmayer H, Sauer M, Marx H (2017) Metabolic Flexibility of *Yarrowia lipolytica* Growing on Glycerol. Front Microbiol 8:1-9. https://doi.org/10.3389/fmicb.2017.00049

European Commission (2023a) REPowerEU: affordable, secure and sustainable energy for Europe. In: https://commission.europa.eu/strategy-and-policy/priorities-2019-2024/european-green-deal/repowereu-affordable-secure-and-sustainable-energy-europe_en. Accessed 19 April 2023

European Commission (2023b) Waste and recycling. In: https://environment.ec.europa.eu/topics/waste-and-recycling_en. Accessed 19 April 2023

Eurostat (2023a) Share of fossil fuels in gross available energy. In: https://ec.europa.eu/eurostat/databrowser/view/NRG_IND_FFGAE__custom_4713488/bookmark/table?lang=en&bookmarkId=b28d8f1d-02c4-4ce5-bb5c-084990ee5a58. Accessed 18 April 2023

Eurostat (2023b) Waste statistics. In: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Waste_statistics#Total_waste_generation. Accessed 18 April 2023

Eurostat (2023c) Fossil fuels stabilised at 70% of energy use in 2021. In: https://ec.europa.eu/eurostat/en/web/products-eurostat-news/w/ddn-20230130-1. Accessed 20 July 2023

Eurostat (2023d) Renewable energy statistics. In: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Renewable_energy_statistics#Share_of_renewable_energy_more_than_doubled_between_2004_and_2021. Accessed 20 July 2023

Expert Market Research (2023) Global Citric Acid Market. 2022. https://www.expertmarketresearch.com/reports/citric-acid-market. Accessed 31 Mar 2023

Fabiszewska AU, Zieniuk B, Kozłowska M, Mazurczak-Zieniuk PM, Wołoszynowska M, Misiukiewicz-Stępień P, Nowak D (2021) Studies on upgradation of waste fish oil to lipid-rich yeast biomass in *Yarrowia lipolytica* batch cultures. Foods 10:436. https://doi.org/10.3390/foods10020436

Fassinou WF, Sako A, Fofana A, Koua KB, Toure S (2010) Fatty acids composition as a means to estimate the high heating value (HHV) of vegetable oils and biodiesel fuels. Energy 35:4949–4954. https://doi.org/10.1016/j.energy.2010.08.030

Fei Q, Chang HN, Shang L, Choi J (2011a) Exploring low-cost carbon sources for microbial lipids production by fed-batch cultivation of *Cryptococcus albidus*. Biotechnology and Bioprocess Engineering 16:482–487. https://doi.org/10.1007/s12257-010-0370-y

Fei Q, Nam H, Shang L, Choi J, Kim N, Kang J (2011b) The effect of volatile fatty acids as a sole carbon source on lipid accumulation by *Cryptococcus albidus* for biodiesel production. Bioresour Technol 102:2695–2701. https://doi.org/10.1016/j.biortech.2010.10.141

Ferreira M, Fernandes H, Peres H, Oliva-Teles A, Belo I, Salgado JM (2021) Polyunsaturated fatty acids production by solid-state fermentation on polyurethane foam by *Mortierella alpina*. Biotechnol Prog 37:e3113. https://doi.org/10.1002/btpr.3113

Ferreira P, Lopes M, Mota M, Belo I (2016) Oxygen mass transfer impact on citric acid production by *Yarrowia lipolytica* from crude glycerol. Biochem Eng J 110:35–42. https://doi.org/10.1016/j.bej.2016.02.001

Fickers P, Benetti P-H, Waché Y, Marty A, Mauersberger S, Smit MS, Nicaud J-M (2005) Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications. FEMS Yeast Res 5:527–543. https://doi.org/10.1016/j.femsyr.2004.09.004

Filippousi R, Antoniou D, Tryfinopoulou P, Nisiotou AA, Nychas G-J, Koutinas AA, Papanikolaou S (2019) Isolation, identification and screening of yeasts towards their ability to assimilate biodiesel-derived crude glycerol: microbial production of polyols, endopolysaccharides and lipid. J Appl Microbiol 127:1080–1100. https://doi.org/10.1111/jam.14373

Fontanille P, Kumar V, Christophe G, Nouaille R, Larroche C (2012) Bioconversion of volatile fatty acids into lipids by the oleaginous yeast *Yarrowia lipolytica*. Bioresour Technol 114:443–449. https://doi.org/10.1016/j.biortech.2012.02.091

Friedlander J, Tsakraklides V, Kamineni A, Greenhagen EH, Consiglio AL, Macewen K, Crabtree D V, Afshar J, Nugent RL, Hamilton MA, Shaw AJ, South CR, Stephanopoulos G, Brevnova EE (2016) Engineering of a high lipid producing *Yarrowia lipolytica* strain. Biotechnol Biofuels 9:77. https://doi.org/10.1186/s13068-016-0492-3

Gaeta-Bernardi A, Parente V (2016) Organic municipal solid waste (MSW) as feedstock for biodiesel production: A financial feasibility analysis. Renew Energy 86:1422–1432. https://doi.org/10.1016/j.renene.2015.08.025

Gajdoš P, Nicaud J-M, Čertík M (2017) Glycerol conversion into a single cell oil by engineered *Yarrowia lipolytica*. Eng Life Sci 17:325–332. https://doi.org/10.1002/elsc.201600065

Gameiro T, Lopes M, Marinho R (2016) Hydrolytic-acidogenic fermentation of organic solid waste for volatile fatty acids production at different solids concentrations and alkalinity addition. Water Air Soil Pollut 227:1–16. https://doi.org/10.1007/s11270-016-3086-6

Gao B, Wang F, Huang L, Liu H, Zhong Y, Zhang C (2021) Biomass, lipid accumulation kinetics, and the transcriptome of heterotrophic oleaginous microalga *Tetradesmus bernardii* under different carbon and nitrogen sources. Biotechnol Biofuels 14:4. https://doi.org/10.1186/s13068-020-01868-9

Gao R, Li Z, Zhou X, Bao W, Cheng S, Zheng L (2020) Enhanced lipid production by *Yarrowia lipolytica* cultured with synthetic and waste-derived high-content volatile fatty acids under alkaline conditions. Biotechnol Biofuels 13:1–16. https://doi.org/10.1186/s13068-019-1645-y

Gao R, Li Z, Zhou X, Cheng S, Zheng L (2017) Oleaginous yeast *Yarrowia lipolytica* culture with synthetic and food waste - derived volatile fatty acids for lipid production. Biotechnol Biofuels 10:1–15. https://doi.org/10.1186/s13068-017-0942-6

Ghaly AE, Dave D, Brooks MS, Budge S (2010) Production of biodiesel by enzymatic transesterification: Review. Am J Biochem Biotechnol 6:54–76. https://doi.org/10.3844/ajbbsp.2010.54.76

Gong X, Wu M, Jiang Y, Wang H (2021) Effects of different temperatures and pH values on volatile fatty acids production during codigestion of food waste and thermal-hydrolysed sewage sludge and subsequent volatile fatty acids for polyhydroxyalkanoates production. Bioresour Technol 333:125149. https://doi.org/10.1016/j.biortech.2021.125149

Gong Z, Zhou W, Shen H, Yang Z, Wang G, Zuo Z, Hou Y, Zhao ZK (2016) Co-fermentation of acetate and sugars facilitating microbial lipid production on acetate-rich biomass hydrolysates. Bioresour Technol 207:102–108. https://doi.org/10.1016/j.biortech.2016.01.122

Goswami L, Tejas Namboodiri MM, Vinoth Kumar R, Pakshirajan K, Pugazhenthi G (2017) Biodiesel production potential of oleaginous *Rhodococcus opacus* grown on biomass gasification wastewater. Renew Energy 105:400–406. https://doi.org/10.1016/j.renene.2016.12.044

Gottardi D, Siroli L, Vannini L, Patrignani F, Lanciotti R (2021) Recovery and valorization of agri-food wastes and by-products using the non-conventional yeast *Yarrowia lipolytica*. Trends Food Sci Technol 115:74–86. https://doi.org/10.1016/j.tifs.2021.06.025

Groenewald M, Boekhout T, Neuvéglise C, Gaillardin C, van Dijck PWM, Wyss M (2014) *Yarrowia lipolytica*: Safety assessment of an oleaginous yeast with a great industrial potential. Crit Rev Microbiol 40:187–206. https://doi.org/10.3109/1040841X.2013.770386

Gujjala LKS, Kumar SPJ, Talukdar B, Dash A, Kumar S, Sherpa KCh, Banerjee R (2019) Biodiesel from oleaginous microbes: opportunities and challenges. Biofuels 10:45–59. https://doi.org/10.1080/17597269.2017.1402587

Hapeta P, Kerkhoven EJ, Lazar Z (2020) Nitrogen as the major factor influencing gene expression in *Yarrowia lipolytica*. Biotechnology Reports 27:e00521. https://doi.org/10.1016/j.btre.2020.e00521

Holechek JL, Geli HME, Sawalhah MN, Valdez R (2022) A global assessment: can renewable energy replace fossil fuels by 2050? Sustainability 14: 4792 https://doi.org/10.3390/su14084792

Huang X-F, Liu J-N, Lu L-J, Peng K-M, Yang G-X, Liu J (2016) Culture strategies for lipid production using acetic acid as sole carbon source by *Rhodosporidium toruloides*. Bioresour Technol 206:141–149. https://doi.org/10.1016/j.biortech.2016.01.073

IEA 2021 Renewables 2021, IEA, Paris. In: https://www.iea.org/reports/renewables-2021. Accessed 20 April 2023

IEA Bioenergy (2023) IEA Bioenergy Task 42 – Global biorefinery status report 2022. https://www.ieabioenergy.com/wp-content/uploads/2022/09/IEA-Bioenergy-Task-42-Global-biorefinery-status-report-2022-220712.pdf. Accessed 20 April 2023

Issariyakul T, Dalai AK (2014) Biodiesel from vegetable oils. Renewable and Sustainable Energy Reviews 31:446–471. https://doi.org/10.1016/j.rser.2013.11.001

Jach ME, Malm A (2022) *Yarrowia lipolytica* as an alternative and valuable source of nutritional and bioactive compounds for humans. Molecules 27:2300. https://doi.org/10.3390/molecules27072300

Jeswani HK, Chilvers A, Azapagic A (2020) Environmental sustainability of biofuels: a review. Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences 476:20200351. https://doi.org/10.1098/rspa.2020.0351

Johnravindar D, Karthikeyan OP, Selvam A, Murugesan K, Wong JWC (2018) Lipid accumulation potential of oleaginous yeasts: A comparative evaluation using food waste leachate as a substrate. Bioresour Technol 248:221–228. https://doi.org/10.1016/j.biortech.2017.06.151

Jones AD, Boundy-Mills KL, Barla GF, Kumar S, Ubanwa B, Balan V (2019) Microbial lipid alternatives to plant lipids. In: Balan V (ed) Microbial Lipid Production: Methods and Protocols. Springer New York, New York, NY, pp 1–32

Karamerou EE, Theodoropoulos C, Webb C (2017) Evaluating feeding strategies for microbial oil production from glycerol by *Rhodotorula glutinis*. Eng Life Sci 17:314–324. https://doi.org/10.1002/elsc.201600073

Karamerou EE, Webb C (2019) Cultivation modes for microbial oil production using oleaginous yeasts – A review. Biochem Eng J 151:107322. https://doi.org/10.1016/j.bej.2019.107322

Katre G, Joshi C, Khot M, Zinjarde S, Ravikumar A (2012) Evaluation of single cell oil (SCO) from a tropical marine yeast *Yarrowia lipolytica* NCIM 3589 as a potential feedstock for biodiesel. AMB Express 2:36. https://doi.org/10.1186/2191-0855-2-36

Katre G, Raskar S, Zinjarde S, Ravi Kumar V, Kulkarni BD, RaviKumar A (2018) Optimization of the in situ transesterification step for biodiesel production using biomass of *Yarrowia lipolytica* NCIM 3589 grown on waste cooking oil. Energy 142:944–952. https://doi.org/10.1016/j.energy.2017.10.082

Kolouchová I, Maťátková O, Sigler K, Masák J, Řezanka T (2016) Lipid accumulation by oleaginous and non-oleaginous yeast strains in nitrogen and phosphate limitation. Folia Microbiol 61:431–438. https://doi.org/10.1007/s12223-016-0454-y

Kolouchová I, Schreiberová O, Sigler K, Masak J, Řezanka T (2015) Biotransformation of volatile fatty acids by oleaginous and non-oleaginous yeast species. FEMS Yeast Res 15. https://doi.org/10.1093/femsyr/fov076

Koutinas AA, Chatzifragkou A, Kopsahelis N, Papanikolaou S, Kookos IK (2014) Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. Fuel 116:566–577. https://doi.org/10.1016/j.fuel.2013.08.045

Kumar LR, Yellapu SK, Tyagi RD, Drogui P (2020) Purified crude glycerol by acid treatment allows to improve lipid productivity by *Yarrowia lipolytica* SKY7. Process Biochemistry 96:165–173. https://doi.org/10.1016/j.procbio.2020.06.010

Kumar LR, Yellapu SK, Tyagi RD, Zhang X (2019) A review on variation in crude glycerol composition, bio-valorization of crude and purified glycerol as carbon source for lipid production. Bioresour Technol 293:122155. https://doi.org/10.1016/j.biortech.2019.122155

Kumar LR, Yellapu SK, Yan S, Tyagi RD, Drogui P (2021) Elucidating the effect of impurities present in different crude glycerol sources on lipid and citric acid production by *Yarrowia lipolytica* SKY7. Journal of Chemical Technology & Biotechnology 96:227–240. https://doi.org/10.1002/jctb.6531

Kuttiraja M, Dhouha A, Tyagi RD (2018) Harnessing the effect of pH on lipid production in batch cultures of *Yarrowia lipolytica* SKY7. Appl Biochem Biotechnol 184:1332–1346. https://doi.org/10.1007/s12010-017-2617-y

Kuttiraja M, Douha A, Valéro JR, Tyagi RD (2016) Elucidating the effect of glycerol concentration and C/N ratio on lipid production using *Yarrowia lipolytica* SKY7. Appl Biochem Biotechnol 180:1586–1600. https://doi.org/10.1007/s12010-016-2189-2

Lazar Z, Dulermo T, Neuvéglise C, Crutz-Le Coq A-M, Nicaud J-M (2014) Hexokinase—A limiting factor in lipid production from fructose in *Yarrowia lipolytica*. Metab Eng 26:89–99. https://doi.org/10.1016/j.ymben.2014.09.008

Ledesma-Amaro R, Nicaud J (2016a) *Yarrowia lipolytica* as a biotechnological chassis to produce usual and unusual fatty acids. Prog Lipid Res 61:40–50. https://doi.org/10.1016/j.plipres.2015.12.001

Ledesma-Amaro R, Nicaud J-M (2016b) Metabolic engineering for expanding the substrate range of *Yarrowia lipolytica*. Trends Biotechnol 34:798–809. https://doi.org/10.1016/j.tibtech.2016.04.010

Lee WS, Chua ASM, Yeoh HK, Ngoh GC (2014) A review of the production and applications of waste-derived volatile fatty acids. Chemical Engineering Journal 235:83–99. https://doi.org/10.1016/j.cej.2013.09.002

Ling Y, Sun L, Wang S, Lin CSK, Sun Z, Zhou Z (2019) Cultivation of oleaginous microalga *Scenedesmus obliquus* coupled with wastewater treatment for enhanced biomass and lipid production. Biochem Eng J 148:162–169. https://doi.org/10.1016/j.bej.2019.05.012

Liu H, Song Y, Fan X, Wang C, Lu X, Tian Y (2021) *Yarrowia lipolytica* as an oleaginous platform for the production of value-added fatty acid-based bioproducts. Front Microbiol 11:608662. https://doi.org/10.3389/fmicb.2020.608662

Liu H-H, Ji X-J, Huang H (2015) Biotechnological applications of *Yarrowia lipolytica*: Past, present and future. Biotechnol Adv 33:1522–1546. https://doi.org/10.1016/j.biotechadv.2015.07.010

Liu J, Yuan M, Liu J-N, Huang X-F (2017a) Bioconversion of mixed volatile fatty acids into microbial lipids by *Cryptococcus curvatus* ATCC 20509. Bioresour Technol 241:645–651. https://doi.org/10.1016/j.biortech.2017.05.085

Liu N, Qiao K, Stephanopoulos G (2016) C Metabolic Flux Analysis of acetate conversion to lipids by *Yarrowia lipolytica*. Metab Eng 38:86–97. https://doi.org/10.1016/j.ymben.2016.06.006

Liu N, Wang Q, Jiang J, Zhang H (2017b) Effects of salt and oil concentrations on volatile fatty acid generation in food waste fermentation. Renew Energy 113:1523–1528. https://doi.org/10.1016/j.renene.2017.07.042

Llamas M, Dourou M, González-Fernández C, Aggelis G, Tomás-Pejó E (2020a) Screening of oleaginous yeasts for lipid production using volatile fatty acids as substrate. Biomass Bioenergy 138:105553. https://doi.org/10.1016/j.biombioe.2020.105553

Llamas M, Magdalena JA, González-Fernández C, Tomás-Pejó E (2020b) Volatile fatty acids as novel building blocks for oil-based chemistry via oleaginous yeast fermentation. Biotechnol Bioeng 117:238–250. https://doi.org/10.1002/bit.27180

Llamas M, Tomás-Pejó E, González-Fernández C (2020c) Volatile fatty acids from organic wastes as novel low-cost carbon source for *Yarrowia lipolytica*. N Biotechnol 56:123–129. https://doi.org/10.1016/j.nbt.2020.01.002

Lopes M, Ara C, Gomes N, Belo I (2009a) The use of olive mill wastewater by wild type *Yarrowia lipolytica* strains: medium supplementation and surfactant presence. Journal of Chemical Technology and Biotechnology 84:533–537. https://doi.org/10.1002/jctb.2075

Lopes M, Gomes AS, Silva CM, Belo I (2018) Microbial lipids and added value metabolites production by *Yarrowia lipolytica* from pork lard. J Biotechnol 265:76–85. https://doi.org/10.1016/j.jbiotec.2017.11.007

Lopes M, Gomes N, Mota M, Belo I (2009b) *Yarrowia lipolytica* growth under increased air pressure: influence on enzyme production. Appl Biochem Biotechnol 159:46–53. https://doi.org/10.1007/s12010-008-8359-0

Lopes M, Miranda SM, Alves JM, Pereira AS, Belo I (2019) Waste cooking oils as feedstock for lipase and lipid-rich biomass production. European Journal of Lipid Science and Technology 121:1800188. https://doi.org/10.1002/ejlt.201800188

Lopes M, Miranda SM, Costa AR, Pereira AS, Belo I (2022) *Yarrowia lipolytica* as a biorefinery platform for effluents and solid wastes valorization – challenges and opportunities. Crit Rev Biotechnol 42:163–183. https://doi.org/10.1080/07388551.2021.1931016

Lytras G, Lytras C, Mathioudakis D, Papadopoulou K, Lyberatos G (2021) Food waste valorization based on anaerobic digestion. Waste Biomass Valorization 12:1677–1697. https://doi.org/10.1007/s12649-020-01108-z

Magdouli S, Brar SK, Blais JF (2018) Morphology and rheological behaviour of *Yarrowia lipolytica*: Impact of dissolved oxygen level on cell growth and lipid composition. Process Biochemistry 65:1–10. https://doi.org/10.1016/j.procbio.2017.10.021

Matatkova O, Gharwalova L, Zimola M, Rezanka T, Masak J, Kolouchova I (2017) Using odd-alkanes as a carbon source to increase the content of nutritionally important fatty acids in *Candida krusei*, *Trichosporon cutaneum*, and *Yarrowia lipolytica*. 2017:8195329. https://doi.org/10.1155/2017/8195329

Miller GL (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem 31:426–428. https://doi.org/10.1021/ac60147a030

Mitrea L, Călinoiu L-F, Teleky B-E, Szabo K, Martău A-G, Ştefănescu B-E, Dulf F-V, Vodnar D-C (2022) Waste cooking oil and crude glycerol as efficient renewable biomass for the production of platform organic chemicals through oleophilic yeast strain of *Yarrowia lipolytica*. Environ Technol Innov 28:102943. https://doi.org/10.1016/j.eti.2022.102943

Monteiro MR, Kugelmeier CL, Pinheiro RS, Batalha MO, da Silva César A (2018) Glycerol from biodiesel production: Technological paths for sustainability. Renewable and Sustainable Energy Reviews 88:109–122. https://doi.org/10.1016/j.rser.2018.02.019

Morales-Palomo S, González-Fernández C, Tomás-Pejó E (2022) Prevailing acid determines the efficiency of oleaginous fermentation from volatile fatty acids. J Environ Chem Eng 10:107354. https://doi.org/10.1016/j.jece.2022.107354

Moreno JF, Oulego P, Collado S, Díaz M (2023) Production of biolipids from volatile fatty acids of sewage sludge by *Yarrowia lipolytica*. Fuel 348:128488. https://doi.org/10.1016/j.fuel.2023.128488

Moretto G, Valentino F, Pavan P, Majone M, Bolzonella D (2019a) Optimization of urban waste fermentation for volatile fatty acids production. Waste Management 92:21–29. https://doi.org/10.1016/j.wasman.2019.05.010

Moretto G, Valentino F, Pavan P, Majone M, Bolzonella D (2019b) Optimization of urban waste fermentation for volatile fatty acids production. Waste Management 92:21–29. https://doi.org/10.1016/j.wasman.2019.05.010

Moza A, Ram NR, Srivastava NK, Nikhil GN (2022) Bioprocessing of low-value food waste to high value volatile fatty acids for applications in energy and materials: A review on process-flow. Bioresour Technol Rep 19:101123. https://doi.org/10.1016/j.biteb.2022.101123

Munir M, Ahmad M, Saeed M, Waseem A, Rehan M, Nizami A-S, Zafar M, Arshad M, Sultana S (2019) Sustainable production of bioenergy from novel non-edible seed oil (*Prunus cerasoides*) using bimetallic impregnated montmorillonite clay catalyst. Renewable and Sustainable Energy Reviews 109:321–332. https://doi.org/10.1016/j.rser.2019.04.029

Nair AS, Sivakumar N (2022) Biodiesel production by oleaginous bacteria *Rhodococcus opacus* PD630 using waste paper hydrolysate. Biomass Convers Biorefin. https://doi.org/10.1007/s13399-021-02135-z

Nambou K, Zhao C, Wei L, Chen J, Imanaka T, Hua Q (2014) Designing of a "cheap to run" fermentation platform for an enhanced production of single cell oil from *Yarrowia lipolytica* DSM3286 as a potential feedstock for biodiesel. Bioresour Technol 173:324–333. https://doi.org/10.1016/j.biortech.2014.09.096

Náthia-Neves G, Berni M, Dragone G, Mussatto SI, Forster-Carneiro T (2018) Anaerobic digestion process: technological aspects and recent developments. International Journal of Environmental Science and Technology 15:2033–2046. https://doi.org/10.1007/s13762-018-1682-2

OECD (2022) OECD-FAO Agricultural Outlook 2022-2031. OECD

Ong KL, Li C, Li X, Zhang Y, Xu J, Lin CSK (2019) Co-fermentation of glucose and xylose from sugarcane bagasse into succinic acid by *Yarrowia lipolytica*. Biochem Eng J 148:108–115. https://doi.org/10.1016/j.bej.2019.05.004

Papanikolaou S, Aggelis G (2011a) Lipids of oleaginous yeasts. Part II: Technology and potential applications. European Journal of Lipid Science and Technology 113:1052–1073. https://doi.org/10.1002/ejlt.201100015

Papanikolaou S, Aggelis G (2011b) Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. European Journal of Lipid Science and Technology 113:1031–1051. https://doi.org/10.1002/ejlt.201100014

Papanikolaou S, Chatzifragkou A, Fakas S, Galiotou-Panayotou M, Komaitis M, Nicaud J-M, Aggelis G (2009) Biosynthesis of lipids and organic acids by *Yarrowia lipolytica* strains cultivated on glucose. European Journal of Lipid Science and Technology 111:1221–1232. https://doi.org/10.1002/ejlt.200900055

Papanikolaou S, Chevalot I, Galiotou-panayotou M, Komaitis M, Marc I, Aggelis G (2007) Industrial derivative of tallow: a promising renewable substrate for microbial lipid, single-cell protein and lipase production by *Yarrowia lipolytica*. Electronic Journal of Biotechnology 10:425–435. https://doi.org/10.2225/vol10-issue3-fulltext-8

Papanikolaou S, Chevalot I, Komaitis M, Marc I, Aggelis G (2002) Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. Appl Microbiol Biotechnol 58:308–312. https://doi.org/10.1007/s00253-001-0897-0

Papanikolaou S, Diamantopoulou P, Blanchard F, Lambrinea E, Chevalot I, Stoforos NG, Rondags E (2020) Physiological characterization of a novel wild-type *Yarrowia lipolytica* strain grown on glycerol: effects of cultivation conditions and mode on polyols and citric acid production. Applied Sciences 10:7373. https://doi.org/10.3390/app10207373

Papanikolaou S, Kampisopoulou E, Blanchard F, Rondags E, Gardeli C, Koutinas AA, Chevalot I, Aggelis G (2017) Production of secondary metabolites through glycerol fermentation under carbon-excess conditions by the yeasts *Yarrowia lipolytica* and *Rhodosporidium toruloides*. European Journal of Lipid Science and Technology 119:1600507. https://doi.org/10.1002/ejlt.201600507

Park GW, Chang HN, Jung K, Seo C, Kim Y-C, Choi JH, Woo HC, Hwang I (2017) Production of microbial lipid by *Cryptococcus curvatus* on rice straw hydrolysates. Process Biochemistry 56:147–153. https://doi.org/10.1016/j.procbio.2017.02.020

Park JO, Liu N, Holinski KM, Emerson DF, Qiao K, Woolston BM, Xu J, Lazar Z, Islam MA, Vidoudez C, Girguis PR, Stephanopoulos G (2019) Synergistic substrate cofeeding stimulates reductive metabolism. Nat Metab 1:643–651. https://doi.org/10.1038/s42255-019-0077-0

Park YK, Dulermo T, Amaro RL, Nicaud JM (2018) Biotechnology for Biofuels Optimization of odd chain fatty acid production by *Yarrowia lipolytica*. Biotechnol Biofuels 11:1–12. https://doi.org/10.1186/s13068-018-1154-4

Patel A, Arora N, Mehtani J, Pruthi V, Pruthi PA (2017) Assessment of fuel properties on the basis of fatty acid profiles of oleaginous yeast for potential biodiesel production. Renewable and Sustainable Energy Reviews 77:604–616. https://doi.org/10.1016/j.rser.2017.04.016

Patel A, Karageorgou D, Rova E, Katapodis P, Rova U, Christakopoulos P, Matsakas L (2020) An overview of potential oleaginous microorganisms and their role in biodiesel and omega-3 fatty acid-based industries. Microorganisms 8:434. https://doi.org/10.3390/microorganisms8030434

Patel A, Mahboubi A, Horváth IS, Taherzadeh MJ, Rova U, Christakopoulos P, Matsakas L (2021a) Volatile Fatty Acids (VFAs) generated by anaerobic digestion serve as feedstock for freshwater and marine oleaginous microorganisms to produce biodiesel and added-value compounds. Front Microbiol 12:614612. https://doi.org/10.3389/fmicb.2021.614612

Patel A, Matsakas L, Rova U, Christakopoulos P (2018) Heterotrophic cultivation of *Auxenochlorella protothecoides* using forest biomass as a feedstock for sustainable biodiesel production. Biotechnol Biofuels 11:169. https://doi.org/10.1186/s13068-018-1173-1

Patel A, Sarkar O, Rova U, Christakopoulos P, Matsakas L (2021b) Valorization of volatile fatty acids derived from low-cost organic waste for lipogenesis in oleaginous microorganisms-A review. Bioresour Technol 321:124457. https://doi.org/10.1016/j.biortech.2020.124457

Pereira AS, Belo I, Lopes M (2022a) Enhancing microbial lipids synthesis for biodiesel production by *Y. lipolytica* W29 from volatile fatty acids: two-stage batch strategies. Applied Sciences 12:8614. https://doi.org/10.3390/app12178614

Pereira AS, Lopes M, Duarte MS, Alves MM, Belo I (2023) Integrated bioprocess of microbial lipids production in *Yarrowia lipolytica* using food-waste derived volatile fatty acids. Renew Energy 202:1470–1478. https://doi.org/10.1016/j.renene.2022.12.012

Pereira AS, Lopes M, Miranda SM, Belo I (2022b) Bio-oil production for biodiesel industry by *Yarrowia lipolytica* from volatile fatty acids in two-stage batch culture 106: 2869-2881. Appl Microbiol Biotechnol. https://doi.org/10.1007/s00253-022-11900-7

Pereira AS, Miranda SM, Lopes M, Belo I (2021) Factors affecting microbial lipids production by *Yarrowia lipolytica* strains from volatile fatty acids: Effect of co-substrates, operation mode and oxygen. J Biotechnol 331:37–47. https://doi.org/10.1016/j.jbiotec.2021.02.014

Poli JS, da Silva MAN, Siqueira EP, Pasa VMD, Rosa CA, Valente P (2014) Microbial lipid produced by *Yarrowia lipolytica* QU21 using industrial waste: A potential feedstock for biodiesel production. Bioresour Technol 161:320–326. https://doi.org/10.1016/j.biortech.2014.03.083

Poontawee R, Limtong S (2020) Feeding strategies of two-stage fed-batch cultivation processes for microbial lipid production from sugarcane top hydrolysate and crude glycerol by the oleaginous red yeast *Rhodosporidiobolus fluvialis.* Microorganisms 8:151. https://doi.org/10.3390/microorganisms8020151

PORTDATA (2020a) Municipal waste generation. https://www.pordata.pt/en/Europe/Municipal+waste+generation-3423. Accessed 14 Oct 2022

PORTDATA (2020b) Recycling rate of municipal waste (%). https://www.pordata.pt/en/Europe/Recycling+rate+of+municipal+waste+(percentage)-3426. Accessed 14 Oct 2022

Qian X, Gorte O, Chen L, Zhang W, Dong W, Ma J, Xin F, Jiang M, Ochsenreither K (2020) Continuous self-provided fermentation for microbial lipids production from acetate by using oleaginous yeasts *Cryptococcus podzolicus* and *Trichosporon porosum*. Renew Energy 146:737–743. https://doi.org/10.1016/j.renene.2019.07.013

Qian X, Zhou X, Chen L, Zhang X, Xin F, Dong W, Zhang W, Ochsenreither K, Jiang M (2021) Bioconversion of volatile fatty acids into lipids by the oleaginous yeast *Apiotrichum porosum* DSM27194. Fuel 290:119811. https://doi.org/10.1016/j.fuel.2020.119811

Qiao K, Imam Abidi SH, Liu H, Zhang H, Chakraborty S, Watson N, Kumaran Ajikumar P, Stephanopoulos G (2015) Engineering lipid overproduction in the oleaginous yeast *Yarrowia lipolytica*. Metab Eng 29:56–65. https://doi.org/10.1016/j.ymben.2015.02.005

Radha P, Prabhu K, Jayakumar A, AbilashKarthik S, Ramani K (2020) Biochemical and kinetic evaluation of lipase and biosurfactant assisted ex novo synthesis of microbial oil for biodiesel production by *Yarrowia lipolytica* utilizing chicken tallow. Process Biochemistry 95:17–29. https://doi.org/10.1016/j.procbio.2020.05.009

Rakicka M, Lazar Z, Dulermo T, Fickers P, Nicaud JM (2015) Lipid production by the oleaginous yeast *Yarrowia lipolytica* using industrial by - products under different culture conditions. Biotechnol Biofuels 8:1–10. https://doi.org/10.1186/s13068-015-0286-z

Rane DV, Pawar PP, Odaneth AA, Lali AM (2021) Microbial oil production by the oleaginous red yeast, *Rhodotorula glutinis* NCIM 3168, using corncob hydrolysate. Biomass Convers Biorefin 13:1987–1997. https://doi.org/10.1007/s13399-021-01298-z

Raut G, Jagtap S, Kumar VR, RaviKumar A (2022) Enhancing lipid content of oleaginous *Yarrowia lipolytica* biomass grown on waste cooking oil and its conversion to biodiesel by statistical optimization. Biomass Convers Biorefin. https://doi.org/10.1007/s13399-022-02610-1

Rodrigues G, Pais C (2000) The influence of acetic and other weak carboxylic acids on growth and cellular death of the Yeast *Yarrowia lipolytica.* Food Technol Biotechnol 38:27–32

Sara M, Brar SK, Blais JF (2016) Lipid production by *Yarrowia lipolytica* grown on biodiesel-derived crude glycerol: optimization of growth parameters and their effects on the fermentation efficiency. RSC Adv 6:90547–90558. https://doi.org/10.1039/C6RA16382C

Sarantou S, Stoforos NG, Kalantzi O, Papanikolaou S (2021) Biotechnological valorization of biodiesel-derived glycerol: Trials with the non-conventional yeasts *Yarrowia lipolytica* and *Rhodosporidium* sp. Carbon Resources Conversion 4:61–75. https://doi.org/10.1016/j.crcon.2020.12.006

Sarris D, Tsouko E, Kothri M, Anagnostou M, Karageorgiou E, Papanikolaou S (2023) Upgrading major waste streams derived from the biodiesel industry and olive mills via microbial bioprocessing with non-conventional *Yarrowia lipolytica* strains. Fermentation 9:251. https://doi.org/10.3390/fermentation9030251

Satya Sagar P, Kommoji S, Jayaraj I, Balakrishnan D, Shaik F, Vucha M, Gnanasekaran L (2023) Lipid bioproduction through optimization of thermal diluted acid pretreatment on native grass using *Yarrowia lipolytica*. Fuel 333:126475. https://doi.org/10.1016/j.fuel.2022.126475

Scherhaufer S, Moates G, Hartikainen H, Waldron K, Obersteiner G (2018) Environmental impacts of food waste in Europe. Waste Management 77:98–113. https://doi.org/10.1016/j.wasman.2018.04.038

Silverman AM, Qiao K, Xu P, Stephanopoulos G (2016) Functional overexpression and characterization of lipogenesis-related genes in the oleaginous yeast *Yarrowia lipolytica*. Appl Microbiol Biotechnol 100:3781–3798. https://doi.org/10.1007/s00253-016-7376-0

Singh D, Sharma D, Soni SL, Sharma S, Kumar Sharma P, Jhalani A (2020) A review on feedstocks, production processes, and yield for different generations of biodiesel. Fuel 262:116553. https://doi.org/10.1016/j.fuel.2019.116553

Soong Y-HV, Coleman SM, Liu N, Qin J, Lawton C, Alper HS, Xie D (2023) Using oils and fats to replace sugars as feedstocks for biomanufacturing: Challenges and opportunities for the yeast *Yarrowia lipolytica.* Biotechnol Adv 65:108128. https://doi.org/10.1016/j.biotechadv.2023.108128

Strazzera G, Battista F, Garcia NH, Frison N, Bolzonella D (2018) Volatile fatty acids production from food wastes for biorefinery platforms: A review. J Environ Manage 226:278–288. https://doi.org/10.1016/j.jenvman.2018.08.039

Szczepańska P, Hapeta P, Lazar Z (2021) Advances in production of high-value lipids by oleaginous yeasts. Crit Rev Biotechnol 42:1–22. https://doi.org/10.1080/07388551.2021.1922353

Tai M, Stephanopoulos G (2013) Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. Metab Eng 15:1–9. https://doi.org/10.1016/j.ymben.2012.08.007

Talebi AF, Tabatabaei M, Chisti Y (2014) BiodieselAnalyzer: a user-friendly software for predicting the properties of prospective biodiesel. Biofuel Research Journal 1:55–57. https://doi.org/10.18331/BRJ2015.1.2.4

Tang J, Wang XC, Hu Y, Pu Y, Huang J, Ngo HH, Zeng Y, Li Y (2019) Nutrients removal performance and sludge properties using anaerobic fermentation slurry from food waste as an external carbon source for wastewater treatment. Bioresour Technol 271:125–135. https://doi.org/10.1016/j.biortech.2018.09.087

Tenodi S, Krčmar D, Agbaba J, Zrnić K, Radenović M, Ubavin D, Dalmacija B (2020) Assessment of the environmental impact of sanitary and unsanitary parts of a municipal solid waste landfill. J Environ Manage 258:110019. https://doi.org/10.1016/j.jenvman.2019.110019

Timoumi A, Guillouet SE, Molina-jouve C, Fillaudeau L, Gorret N (2018) Impacts of environmental conditions on product formation and morphology of *Yarrowia lipolytica*. Appl Microbiol Biotechnol 102:3831–3848. https://doi.org/10.1007/s00253-018-8870-3

Tsigie YA, Huynh LH, Ahmed IN, Ju Y-H (2012a) Maximizing biodiesel production from *Yarrowia lipolytica* Po1g biomass using subcritical water pretreatment. Bioresour Technol 111:201–207. https://doi.org/10.1016/j.biortech.2012.02.052

Tsigie YA, Wang C, Kasim NS, Diem Q, Huynh L, Ho Q, Truong C, Ju Y (2012b) Oil production from *Yarrowia lipolytica* Po1g using rice bran hydrolysate. J Biomed Biotechnol 2012:378384. https://doi.org/10.1155/2012/378384

UNEP (2021) Food Waste Index Report 2021. Nairobi

Usmani Z, Sharma M, Awasthi AK, Sharma GD, Cysneiros D, Nayak SC, Thakur VK, Naidu R, Pandey A, Gupta VK (2021) Minimizing hazardous impact of food waste in a circular economy – Advances in resource recovery through green strategies. J Hazard Mater 416:126154. https://doi.org/10.1016/j.jhazmat.2021.126154

Vajpeyi S, Chandran K (2015) Microbial conversion of synthetic and food waste-derived volatile fatty acids to lipids. Bioresour Technol 188:49–55. https://doi.org/10.1016/j.biortech.2015.01.099

van der Walt JP, von Arx JA (1980) The yeast genus *Yarrowia* gen. nov. Antonie Van Leeuwenhoek 46:517–521. https://doi.org/10.1007/BF00394008

Vasaki M, Sithan M, Ravindran G, Paramasivan B, Ekambaram G, Karri RR (2022) Biodiesel production from lignocellulosic biomass using *Yarrowia lipolytica*. Energy Conversion and Management: X 13:100167. https://doi.org/10.1016/j.ecmx.2021.100167

Vasconcelos B, Teixeira JC, Dragone G, Teixeira JA (2019) Oleaginous yeasts for sustainable lipid production—from biodiesel to surf boards, a wide range of "green" applications. Appl Microbiol Biotechnol 103:3651–3667. https://doi.org/10.1007/s00253-019-09742-x

Vastaroucha E-S, Maina S, Michou S, Kalantzi O, Pateraki C, Koutinas AA, Papanikolaou S (2021) Bioconversions of biodiesel-derived glycerol into sugar alcohols by newly isolated wild-type *Yarrowia lipolytica* strains. Reactions 2:499–513. https://doi.org/10.3390/reactions2040032

Vong WC, Au Yang KLC, Liu S-Q (2016) Okara (soybean residue) biotransformation by yeast *Yarrowia lipolytica*. Int J Food Microbiol 235:1–9. https://doi.org/10.1016/j.ijfoodmicro.2016.06.039

Vu DH, Wainaina S, Taherzadeh MJ, Åkesson D, Ferreira JA (2021) Production of polyhydroxyalkanoates (PHAs) by *Bacillus megaterium* using food waste acidogenic fermentation-derived volatile fatty acids. Bioengineered 12:2480–2498. https://doi.org/10.1080/21655979.2021.1935524

Wainaina S, Awasthi MK, Horváth IS, Taherzadeh MJ (2020) Anaerobic digestion of food waste to volatile fatty acids and hydrogen at high organic loading rates in immersed membrane bioreactors. Renew Energy 152:1140–1148. https://doi.org/10.1016/j.renene.2020.01.138

Wierzchowska K, Zieniuk B, Nowak D, Fabiszewska A (2021) Phosphorus and nitrogen limitation as a part of the strategy to stimulate microbial lipid biosynthesis. Applied Sciences 11:11819. https://doi.org/10.3390/app112411819

Xu X-H, Liu Z-X, Shi X-Y, Miao C, Sheng S, Xu Y, Wu F-A, Wang J (2018) Fed-batch fermentation of *Yarrowia lipolytica* using defatted silkworm pupae hydrolysate: a dynamic model-based approach for high yield of lipid production. Waste Biomass Valorization 9:2399–2411. https://doi.org/10.1007/s12649-017-0180-y

Xu Y, Wang X, Li Z, Cheng S, Jiang J (2022) Potential of food waste hydrolysate as an alternative carbon source for microbial oil synthesis. Bioresour Technol 344:126312. https://doi.org/10.1016/j.biortech.2021.126312

Yang R, Chen Z, Hu P, Zhang S, Luo G (2022) Two-stage fermentation enhanced single-cell protein production by *Yarrowia lipolytica* from food waste. Bioresour Technol 361:127677. https://doi.org/10.1016/j.biortech.2022.127677

Yaşar F (2020) Comparision of fuel properties of biodiesel fuels produced from different oils to determine the most suitable feedstock type. Fuel 264:116817. https://doi.org/10.1016/j.fuel.2019.116817

Yasser Farouk R, Mostafa E, Wang Y (2023) Evaluation of hydrogen and volatile fatty acids production system from food waste. Biomass Convers Biorefin 13:5253–5259. https://doi.org/10.1007/s13399-021-02056-x

Yen H-W, Zhang Z (2011) Effects of dissolved oxygen level on cell growth and total lipid accumulation in the cultivation of *Rhodotorula glutinis*. J Biosci Bioeng 112:71–74. https://doi.org/10.1016/j.jbiosc.2011.03.013

Zeng S, Liu H, Shi T, Song P, Ren L, Huang H (2018) recent advances in metabolic engineering of *Yarrowia lipolytica* for lipid overproduction. European Journal of Lipid Science and Technology 120:1–10. https://doi.org/10.1002/ejlt.201700352

Zhang L, Loh K-C, Kuroki A, Dai Y, Tong YW (2021) Microbial biodiesel production from industrial organic wastes by oleaginous microorganisms: Current status and prospects. J Hazard Mater 402:123543. https://doi.org/10.1016/j.jhazmat.2020.123543

Zhang S, Jagtap SS, Deewan A, Rao C V (2019a) pH selectively regulates citric acid and lipid production in *Yarrowia lipolytica* W29 during nitrogen-limited growth on glucose. J Biotechnol 290:10–15. https://doi.org/10.1016/j.jbiotec.2018.10.012

Zhang X, Chen J, Wu D, Li J, Tyagi RD, Surampalli RY (2019b) Economical lipid production from *Trichosporon oleaginosus* via dissolved oxygen adjustment and crude glycerol addition. Bioresour Technol 273:288–296. https://doi.org/10.1016/j.biortech.2018.11.033

Zheng Y, Chi Z, Ahring BK, Chen S (2012) Oleaginous yeast *Cryptococcus curvatus* for biofuel production: Ammonia's effect. Biomass Bioenergy 37:114–121. https://doi.org/10.1016/j.biombioe.2011.12.022