Universidade do Minho
Escola de Engenharia

Ana Rita Quintela da Costa

Exploring *Yarrowia* species as cellular platforms for developing circular economy in agro-food industries of vegetable oils production

December 2023

Exploring *Yarrowia* species as cellular platforms for developing circular economy in agro-food industries of vegetable oils production

Ana Rita Quintela da Costa

UMinho | 2023

Universidade do Minho
Escola de Engenharia

Ana Rita Quintela da Costa

Exploring *Yarrowia* species as cellular platforms for developing circular economy in agro-food industries of vegetable oils production

Doctoral Thesis
Doctoral Program in Chemical and Biological Engineering

Work developed under the supervison of:
Professor Doutor Isabel Maria Pires Belo
Doctor José Manuel Salgado Seara

December 2023

ACKNOWLEDGEMENTS

Após estes anos de trabalho não posso deixar de lembrar todas as pessoas que de alguma forma contribuíram para a concretização desta etapa. Deixo aqui os meus sinceros agradecimentos:

Aos meus orientadores Profª Doutora Isabel Belo e Doutor José Salgado pela oportunidade de trabalhar neste projeto e por me terem recebido tão bem no seu laboratório. Obrigada por todo o apoio, paciência, disponibilidade e partilha de conhecimentos.

À Doutora Marlene Lopes pela disponibilidade e apoio na escrita de artigos científicos.

Ao Doutor David Outeiriño do Departamento de Engenharia Química da Universidade de Vigo pela sua contribuição no pré-tratamento do bagaço de azeitona com líquidos iónicos.

À Helena Fernandes pela sua preciosa ajuda, principalmente nos meus últimos meses de trabalho no laboratório. Obrigada pelo teu apoio, carinho e palavras de incentivo. Às restantes "Labólogas" Bruna Dias, Marta Ferreira, Sílvia Fernandes, Sílvia Miranda e Sofia Pereira por todo o apoio, incentivo e pelo espírito de entreajuda incrível que sempre se viveu no laboratório. Obrigada por todos os bons momentos que passamos e por tornarem toda esta jornada mais fácil.

A todos os que passaram pelo Laboratório de Bioprocessos e Biossistemas e contribuíram para o bom ambiente de trabalho e espírito de entreajuda, em particular ao Diogo Filipe, Daniel Sousa, Joana Oliveira, Patrícia Dias e Patrícia Ferreira. À Paulina Leite agradeço a sua ajuda com o reator de SSF.

À Leslie Amaral pela amizade, apoio, incentivo e momentos de descontração nas minhas visitas ao Departamento de Biologia. Obrigada por continuares a fazer parte desta grande aventura.

A todos os membros da minha família pelo apoio, preocupação e interesse pelo meu trabalho. Deixo um agradecimento especial aos meus pais e ao Pedro por sempre me incentivarem a continuar a estudar e pelos sacrifícios que fizeram para que isso se concretizasse. Obrigada pelo apoio, carinho e compreensão que demonstraram, não só nestes quatro anos, mas desde sempre.

Ao Paulo pela amizade, paciência, amor e compreensão. Obrigada pelas palavras de incentivo, por sempre acreditares em mim e estares ao meu lado nos momentos mais difíceis. O teu apoio foi fundamental ao longo destes anos.

Ao Centro de Engenharia Biológica da Universidade do Minho, instituição de acolhimento, por ter garantido todas as condições para a realização deste trabalho. À Fundação para a Ciência e Tecnologia (FCT) pela atribuição da bolsa de doutoramento (SFRH/BD/139098/2018).

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or 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

Exploração de espécies de *Yarrowia* como plataformas celulares para o desenvolvimento de uma economia circular em indústrias agroalimentares de produção de óleos vegetais

As plantas oleaginosas são cultivadas para obtenção de óleos vegetais e do processo de extração destes óleos resultam grandes quantidades de subprodutos, designados de bagaços de oleaginosas, que geralmente são usados como alimento animal ou para a produção de energia. No entanto, dadas algumas limitações destas aplicações, o desenvolvimento de novas estratégias de valorização destes subprodutos é de extrema relevância, nomeadamente através de processos biotecnológicos que envolvem a produção de compostos de valor acrescentado. Neste trabalho, foi estudado o potencial de valorização de bagaços de girassol, colza e azeitona através do seu uso como substratos para a produção de enzimas por espécies de *Yarrowia* em fermentação em estado sólido (FES). Inicialmente, foi usado um desenho experimental *simplex-centroid* para identificar a mistura ótima para a produção de lipase e protease pela *Y. lipolytica* W29 através de FES. Uma atividade máxima de lipase de (102 ± 17) U/g foi obtida com uma mistura de partes iguais em massa de bagaço de girassol e azeitona e, em contraste, uma mistura dos três bagaços maximiza a atividade de protease num valor de (63 ± 3) U/g. Seguidamente, foram testados vários pré-tratamentos biológicos e físicos aos substratos antes da FES com *Y. lipolytica* W29 para a produção de lipase. Os resultados mais promissores foram obtidos com a radiação micro-ondas, que aumentou a atividade da lipase e o crescimento da levedura em 17% e 44%, respetivamente, em comparação com a mistura não tratada. O aumento de escala das condições ótimas para a produção de lipase foi realizado, aumentando até 40 vezes a quantidade de substrato utilizado e recorrendo a diferentes tipos de biorreatores usados em FES. A atividade de lipase mais elevada foi obtida em reatores do tipo tabuleiro, sendo que a disponibilidade de oxigénio teve um papel relevante na produção da enzima. Por outro lado, a utilização de um biorreactor de tambor horizontal com agitação e arejamento, resultou num atraso no pico da lipase e melhorou significativamente o crescimento da levedura. Sendo o bagaço de azeitona um dos bagaços sem aplicação estabelecida quer económica quer ambientalmente, foram estudadas outras estratégias para a valorização. Pré-tratamentos hidrotérmicos e com líquidos iónicos resultaram em alterações na composição do bagaço, aumentando a libertação de açucares durante a hidrólise enzimática comparado com o material não tratado. *Yarrowia lipolytica* W29 foi capaz de crescer nos hidrolisados, resultando na produção de biomassa de levedura com valor máximo de 10 g/L. Esta biomassa tem aplicações como alimento, dado que *Y. lipolytica* tem uma elevada percentagem de proteína que pode chegar aos 45% e está aprovada como segura para alimentação humana.

Palavras-chave: bagaços de oleaginosas; fermentação em estado sólido; lipase; *Yarrowia lipolytica*

ABSTRACT

Exploring *Yarrowia* species as cellular platforms for developing circular economy in agro-food industries of vegetable oil production

Oilseed crops are cultivated for the production of vegetable oils and the extraction of these oils results in the production of high volumes of by-products, designated as oil cakes, which are generally used for animal feed or energy production. Since the current applications are associated with some limitations, development of new strategies for oil cakes valorization is highly relevant, namely through biotechnological processes for value-added compounds production. In the present work, the potential of sunflower, rapeseed and olive cakes valorization was studied through their use as substrates in solid state fermentation (SSF) for enzyme production by *Yarrowia* species. Firstly, a simplex-centroid experimental design was used to identify the optimum substrate mixture for lipase and protease production by *Y. lipolytica* W29 in SSF. Maximum lipase activity of (102 ± 17) U/g was attained with equal parts of sunflower and olive cakes and, on the other hand, a mixture of the three oil cakes led to a maximum protease production of (63 ± 3) U/g. Then, several biological and physical pretreatments were tested before SSF with *Y. lipolytica* W29 for lipase production. The most promising results were obtained with microwave irradiation, which increased lipase activity and yeast growth in 17% and 44%, respectively, compared to SSF with the untreated substrate. Scale-up of the optimum conditions for lipase production was performed with different substrate loadings (increasing up to 40-fold) and bioreactor designs used in SSF. Higher lipase activity was achieved in tray bioreactors and oxygen availability played a major role in the production of this enzyme. Conversely, the use of a horizontal drum bioreactor with agitation and forced aeration resulted in a delay on the lipase peak and significantly improved yeast growth. Since olive cakes are considered by-products without an established economical or environmental application, other valorization strategies were further studied. Hydrothermal and ionic liquids pretreatments resulted in changes in the composition of the oil cake and, consequently, increased sugar release during enzymatic hydrolysis compared to the untreated material. *Yarrowia lipolytica* W29 was able to grow in the hydrolysates, essentially resulting in yeast biomass production, reaching a maximum value of 10 g/L. This biomass has applications as a food ingredient since *Y. lipolytica* as a high protein content that can reach 45% and this yeast is considered safe for human consumption.

Keywords: lipase; oil cakes; solid state fermentation; *Yarrowia lipolytica*

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

4-NPB	4-nitrophenyl butyrate
4-NPL	4-nitrophenyl laurate
4-NPP	4-nitrophenyl palmitate
ADF	Acid detergent fibre
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BSA	Bovine serum albumin
C/N	Carbon/nitrogen
CECT	Colección Española de Cultivos Tipo
CMC	Carboxymethylcellulose
COVID-19	Coronavirus disease 2019
CSL	Corn steep liquor
DNS	Dinitrosalicylic
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FID	Flame ionized detector
GC	Gas chromatography
GRAS	Generally regarded as safe
HPLC	High-performance liquid chromatography
ILs	Ionic liquids
ND	Not detected
NDF	Neutral detergent fibre
OC	Olive cake
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
OMWW	Olive mill wastewater
PDA	Potato dextrose agar
RC	Rapeseed cake
RI	Refractive index
rpm	Revolutions per minute

SC	Sunflower cake
SD	Standard deviation
SmF	Submerged fermentation
SSF	Solid state fermentation
UN	United Nations
UV	Ultraviolet
YNB	Yeast Nitrogen Base
YPD	Yeast extract, peptone, dextrose
YPDA	Yeast extract, peptone, dextrose, agar

REMARKS

In general, the International System of Units (SI) was used in this work. Sometimes multiples and submultiples of the SI units were also used, as well as other non-SI units but allowed by SI, such as the use of liter to express volume. Some units not recognized by the SI were also used to express some variables, such as volume percentage (% v/v), mass percent (% w/w), and weight per volume percent (% w/v) to denote the composition of some solutions and the revolutions per minute (rpm) to indicate the agitation rates.

1 MOTIVATION AND OUTLINE

This chapter introduces the background information about the theme of the work and its objectives. The outline of the thesis and its outputs are also presented.

1.1 CONTEXT AND MOTIVATION

World population reached 8 billion in November of 2022 and is expected to reach 9.7 billion by the year of 2050 (UN, 2022). The increase in human population is associated with a higher demand for agricultural commodities, which, in the next decade, is expected to grow at a rate of 1.1% each year (OECD-FAO, 2022).

Oilseeds are included in these major commodities and the oil extracted from these crops is mainly used for human consumption and biodiesel production (OECD-FAO, 2022). Even though growth of vegetable oil demand for these applications is slowing down compared to the last decade (OECD-FAO, 2022), high amounts of by-products, named oil cakes, will be increasingly produced every year. Valorization of oil cakes, for instance, obtained during sunflower and rapeseed oil extraction, often includes their utilization as animal feed since these materials are generally rich in protein. Nonetheless, the presence of antinutritional factors may have a negative impact on animal health and growth performance (Arrutia et al., 2020). Likewise, strategies to increase their nutritional and/or economical value should be investigated.

Olive oil production occurs mainly in Mediterranean countries using a two-phase centrifugation system, which leads to the production of olive oil and a semi-solid by-product with high moisture content (Gullón et al., 2020). These by-products are produced in a short period of time and their handling and disposal can be challenging for the olive mills. Since the low protein and high fiber contents of these by-products unable their use in animal feed formulations, after olive oil extraction, their residual oil is extracted with solvents and the remaining solids are burned for energy production (Gullón et al., 2020; Peri, 2014). Since this approach is energy demanding and can negatively affect the environment, identification of alternative valorization options for these underexplored by-products is also fundamental.

The fact that these materials are rich in polysaccharides, such as cellulose and hemicellulose and in lipids, minerals and, in some cases, protein makes them promising substrates for microbial growth (Ravindran et al., 2018). The microorganisms added to these materials can metabolize the substrate' components, resulting in substrate biotransformation and the release of bioactive molecules with industrial interest (Sousa et al., 2023). Thus, value-added compounds production can be achieved at low costs in eco-friendly bioprocesses while oil cakes can be further explored and valorized (Soccol et al., 2017). *Yarrowia lipolytica* is a non-conventional and non-pathogenic yeast widely used in biotechnological bioprocesses due to its ability to convert an extensive variety of substrates into value-added compounds (Lopes et al., 2022). Moreover, the biomass of this microorganism is considered safe for human consumption, being a promising alternative to animal protein (Groenewald et al., 2014). Although the

majority of reports employs this microorganism in submerged fermentations, the fact that *Y. lipolytica* is a dimorphic yeast makes it a good candidate for fermentations in solid state.

The main objective of this thesis is to study the potential to up-grade by-products from vegetable oil industries, namely oil cakes, using biotechnological processes with low environmental impact, following the concept of circular economy. For this purpose, oil cakes from olive, sunflower and rapeseed oil extraction will be employed in solid state fermentation (SSF) with *Yarrowia* species to produce biocompounds with industrial interest, with focus on lipase production. The impact of biological and physical pretreatments on lipase and yeast biomass production was assessed and scale-up of the optimum SSF conditions for lipase production was performed using trays and horizontal drum bioreactors. Finally, the influence of olive cake pretreatments on sugar release during enzymatic hydrolysis was examined and the obtained hydrolysates were used as culture medium for *Y. lipolytica* growth.

1.2 Outline of the Thesis

This thesis is divided into seven chapters:

In **Chapter 1**, the current chapter, are outlined the motivation and the main goals of this work. The structure and the outputs of this thesis are also highlighted.

In **Chapter 2** is presented a review on the state of the art of the by-products produced in vegetable oil industries, their current applications and the alternative and eco-friendly bioprocesses used for their valorization. Additionally, the utilization of *Y. lipolytica* in these bioprocesses for substrate biotransformation and value-added compounds production is discussed.

In **Chapter 3** the potential of oil cakes as substrates for SSF with *Y. lipolytica* was evaluated. The optimum mixture of oil cakes for lipase and protease production by *Y. lipolytica* W29 was defined using an experimental design.

In **Chapter 4** the optimum substrate mixture for lipase production was submitted to biological and physical pretreatments to increase sugars availability and substrate accessibility before SSF. The effect of pretreatment on lipase and yeast biomass production was evaluated.

In **Chapter 5** the scale up of SSF for lipase production by *Y. lipolytica* was performed. Additionally, the effect of different bioreactor designs and oxygen availability on lipase production and yeast growth was also studied.

In **Chapter 6** fractioning of olive cake was performed using ionic liquids and hydrothermal pretreatments. The hydrolysates obtained after enzymatic hydrolysis were used as culture medium in batch cultures with *Y. lipolytica*.

In **Chapter 7** are presented the main conclusions and future perspectives of this 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

Costa, A. R.; Fernandes, Helena; Salgado, J. M.; Belo, I. "Solid state and semi-solid fermentations of olive oil and sunflower cakes with *Yarrowia lipolytica*: impact of biological and physical pretreatments" submitted to Fermentation (July 2023)

Costa, A.R., Salgado, J.M., Belo, I., 2023. Olive and sunflower cakes as suitable substrates for lipase production by *Yarrowia* spp.: from flasks to bioreactor. Biocatal. Agric. Biotechnol. 51, 102783. https://doi.org/10.1201/9781420077070

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. Crit. Rev. Biotechnol. 42, 163–183. https://doi.org/10.1080/07388551.2021.1931016

Costa, A.R., Salgado, J.M., Lopes, M., Belo, I., 2022. Valorization of by-products from vegetable oil industries: Enzymes production by *Yarrowia lipolytica* through solid state fermentation. Front. Sustain. Food Syst. 6. https://doi.org/10.3389/fsufs.2022.1006467

Leite, P., Sousa, D., Fernandes, H., Ferreira, M., Costa, A.R., Filipe, D., Gonçalves, M., Peres, H., Belo, I., Salgado, J.M., 2021. Recent advances in production of lignocellulolytic enzymes by solid-state fermentation of agro-industrial wastes. Curr. Opin. Green Sustain. Chem. 27, 100407. https://doi.org/10.1016/j.cogsc.2020.100407

POSTER PRESENTATION

Costa, A.R.; Salgado, J.M.; Belo, I. (2022) "Solid state fermentation of oil cakes with *Yarrowia lipolytica* for lipase production". Poster presentation at the 3rd BioIberoAmerica, Ibero-American Congress on Biotechnology, Braga, Portugal

Costa, A.R.; Salgado, J.M.; Belo, I. (2021) "Valorization of by-products from vegetable oils production: enzyme production by *Yarrowia lipolytica* through solid state fermentation". Poster presentation at II Chemical & Biological Engineering Doctoral Symposium, Braga, Portugal. ***Best poster prize awarded by The American Society of Microbiology.***

1.4 REFERENCES

Arrutia, F., Binner, E., Williams, P., Waldron, K.W., 2020. Oilseeds beyond oil: Press cakes and meals supplying global protein requirements. Trends Food Sci. Technol. 100, 88–102. https://doi.org/10.1016/j.tifs.2020.03.044

Groenewald, M., Boekhout, T., Neuvéglise, C., Gaillardin, C., Van Dijck, P.W.M., 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

Gullón, P., Gullón, B., Astray, G., Carpena, M., Fraga-Corral, M., Prieto, M.A., Simal-Gandara, J., 2020. Valorization of by-products from olive oil industry and added-value applications for innovative functional foods. Food Res. Int. 137. https://doi.org/10.1016/j.foodres.2020.109683

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. Crit. Rev. Biotechnol. 42, 163–183. https://doi.org/10.1080/07388551.2021.1931016

OECD-FAO, 2022. Oilseeds and Oilseed Products. OECD-FAO Agric. Outlook 2022 – 2031 127–138. https://doi.org/10.1787/agr_outlook-2018-7-en

Peri, C., 2014. The olive oil refining process, in: The Extra-Virgin Olive Oil Handbook. pp. 201–210. https://doi.org/10.1002/9781118460412.ch17

Ravindran, R., Hassan, S., Williams, G., Jaiswal, A., 2018. A Review on Bioconversion of Agro-Industrial Wastes to Industrially Important Enzymes. Bioengineering 5, 93. https://doi.org/10.3390/bioengineering5040093

Soccol, C.R., Costa, E.S.F., Letti, L.A.J., Karp, S.G., Vandenberghe, L.P. de S., Woiciechowski, A.L., 2017. Recent developments and innovations in solid state fermentation. Biotechnol. Res. Innov. 1, 52–71. https://doi.org/10.1016/j.biori.2017.01.002

Sousa, D., Salgado, J.M., Cambra-López, M., Dias, A., Belo, I., 2023. Biotechnological valorization of oilseed cakes: Substrate optimization by simplex centroid mixture design and scale-up to tray bioreactor. Biofuels, Bioprod. Biorefining 17, 121–134. https://doi.org/10.1002/bbb.2428

UN, 2022. World Population Prospects 2022: Summary of Results. United Nations Dep. Econ. Soc. Aff. Popul. Div.

2 LITERATURE REVIEW

Oil cakes, by-products of vegetable oil extraction processes, are produced in large quantities every year and have been proposed for biotechnological approaches of new routes of valorization, including their use as substrates for solid state and submerged fermentations. *Yarrowia lipolytica* is an oleaginous yeast with industrial interest due to its ability to metabolize a wide range of substrates and produce several value-added compounds. This feature has been extensively studied in SmF processes, however, some recent studies have reported promising results in SSF processes.

In this Chapter, the valorization of oil cakes from olive, sunflower and rapeseed oil extraction via solid state fermentation (SSF) and submerged fermentation (SmF) is discussed with focus on olive cake (OC), an underexploited by-product largely produced in Portugal and other Mediterranean countries. Production of bioactive compounds and substrate biotransformation by *Y. lipolytica* is also presented.

2.1 VEGETABLE OIL INDUSTRIES

Vegetable oils are extracted from seeds, nuts or fruits and are generally characterized by a high content in triacylglycerols and, to a lesser extent, free fatty acids, mono - and diacylglycerols, sterols and tocopherols (Mailer, 2016; Savva and Kafatos, 2016). However, their chemical composition can be affected by the type of plant and its genetic variation, climate, growing conditions and the level of oil refinement. Vegetable oils can be classified as edible when the oils are safe for human consumption and these may include olive, rapeseed, flaxseed, sunflower and soybean oils (Savva and Kafatos, 2016). In contrast, non-edible vegetable oils, for instance, jatropha and castor oils, are used for the production of biodiesel, lubricants, cosmetics and detergents (Jaswanth et al., 2022).

Olive oil is an example of edible oil and its production is observed predominantly in Mediterranean countries, being Spain the leading producer, followed by Italy, Greece and Portugal. Moreover, in the crop year of 2021/22, 2.3 million tons of olive oil were produced in these countries, accounting for 70% of the olive oil produced worldwide (European Comission, 2023). The first method implemented for olive oil extraction, usually named traditional discontinuous system, was performed by hydraulic pressing and, by the end of this process, three streams are obtained: a solid by-product named olive cake (OC), olive oil and, since water was added to the olives to facilitate oil extraction, olive mill wastewater (OMWW) (Dermeche et al., 2013). Nowadays, this method is limited to some small olive mills and continuous systems with phase separation performed by centrifugation are commonly used (Contreras et al., 2020). In the continuous three-phase system, water is added to the olive paste also resulting in the production of three streams, two by-products (OC and OMWW) and olive oil, generating higher amounts of OMWW than in the traditional method. In particular, in the traditional system 40 – 60 L of OMWW are produced per 100 kg of olives, while in the three-phase system between 80 and 120 L are produced during the processing of the same amount of olives (Dermeche et al., 2013). The high production of OMWW and the fact that this by-product presents environmental risks due to its high content of organic matter and phenolic compounds (Amaral et al., 2008) led to the development of a two-phase centrifugation system, where only two streams are produced: olive oil and OC. The two phase-system is considered an eco-friendly extraction process since low volumes of water is consumed and low wastewater is produced. Moreover, using this extraction system, for each 1000 kg of processed olives, around 200 kg of olive oil is recovered and between 800 and 950 kg of OC is produced (Azbar et al., 2004). As stated so far, regardless of the method employed, olive oil extraction leads to the production of a solid or semi-solid by-product with varying moisture content. In particular, a moisture content between 25 and 30% is found in OC from the traditional system while the three and two-phase systems results in a by-product with a

content of water around 45 – 60% and 50 – 70%, respectively. The high content of water, particularly in OC obtained in the two-phase system, makes the storage, handling and valorization of this material very challenging (Gullón et al., 2020).

Oilseeds are mainly cultivated for the oil present in their seeds and 65% of these vegetable oils are used for human consumption, while a smaller fraction (15%) is used for biodiesel production. Furthermore, these oils also have applications in cosmetic and feed industries and in the production of varnishes (Mailer, 2016; OECD-FAO, 2022). However, in some cases, the oil is considered a by-product since the materials obtained after oil extraction have a higher value compared to the oil. For instance, soybean is mostly cultivated for animal feed due to its high percentage of protein and cotton for its fiber (Mailer, 2016). Soybean, mainly cultivated in Brazil and United States, is the major oilseed crop produced worldwide, followed by rapeseed and sunflower. Production of these oilseeds are expected to increase in the next years, with soybean production reaching 411 million tons by 2031. Rapeseed, sunflower and groundnuts will grow at slower rate compared to the last decade and are expected to reach 188 million tons by the same year. Expansion of soybean cultivation in South America is associated with a negative environmental impact due to Amazon deforestation. Furthermore, in European Union, consumers concerns related to the used of genetically modified by-products in animal feed have been raising, which could reduce the demand for soybean and related by-products in these countries. Thus, exploitation of other oilseeds crops, such as rapeseed and sunflower, which are already produced in European countries, could reduce the dependence on soybean imports (OECD-FAO, 2022, 2021).

Furthermore, more than 90% of oilseeds produced worldwide are crushed for oil recovery and only a small amount are used for direct consumption, leading to the production of high amounts of solid by-products. Oil cakes are used for animal feed and its consumption has been growing in the last decade, a tendency that is expected to continue in the next years. Due to COVID-19, an increase in prices of oil cakes, including by-products from soybean, sunflower and rapeseed oil extraction, were registered in the second half of 2020 and continued to raise through 2021, reaching values above 400 USD per ton. However, in the next years, a reduction in prices is expected after the recovery from the effects of COVID-19 (OECD-FAO, 2022).

Traditionally, oil extraction from oilseeds is performed either by mechanical pressing or with organic solvents, which maximizes oil recovery. The term “meal” is often used to designate the by-products from oilseeds extraction with solvents. On the other hand, the by-products resultant from pressing are referred to as “cakes”, however, there is a clear ambiguity associated with the utilization of both terms (Ancuţa and Sonia, 2020; Arrutia et al., 2020).

2.2 OIL CAKES COMPOSITION AND THEIR TRADITIONAL APPLICATIONS

The composition of oil cakes is very diverse and is mostly dependent on the plant variety, growing conditions and the oil extraction method employed (Ancuţa and Sonia, 2020; Ramachandran et al., 2007). Table 2.1 shows the composition of oil cakes resultant from olive oil extraction and from two major oilseed crops, sunflower cake (SC) and rapeseed cake (SC). Ash percentage in these oil cakes is below 10% (w/w) and, regarding mineral content, the three oil cakes are rich in calcium (Feng and Zuo, 2015; Leite et al., 2016; Petraru et al., 2021) and OC also presents high content in potassium (Leite et al., 2016). Other minerals such as zinc, copper and magnesium can also be found in these by-products. The oil cake obtained during olive oil production is characterized by a high content in residual oil since the extraction process does not involve the use of solvents. Regarding SC and RC, the percentage of lipids in these by-products presents higher variability among the studies reported and these differences are mostly related with the extraction process, since the use of solvents results in oil cakes with lower residual oil content (Ancuţa and Sonia, 2020). These oil cakes are characterized by a high content of oleic (18:1) and linoleic (18:2) acids and the unsaturated fatty acids account for more than 85% of total fatty acids (Antónia Nunes et al., 2018; Gao et al., 2023; Petraru et al., 2021). While OC is characterized by a low crude protein content, oil cakes obtained during sunflower and rapeseed oil extraction have high percentage of crude protein, which accounts, in some cases, for 40% (w/w) on dry matter. Taking into account the content of lignin, cellulose and hemicellulose, the highest fiber content can be found in OC, with a highlight to the percentage of lignin, which is significantly higher in this by-product compared to SC and RC.

Table 2.1: Chemical characterization of oil cakes.

Parameters (%)	**OC**[1-6]	**RC**[7-11]	**SC**[7,12-14]
Ash	3.4 - 9.4	5.0 – 7.3	5.26 - 8.84
Lipids	5.8 - 17	2.04 – 15.3	2.34 – 15.77
Crude protein	4.3 – 7.4	31.84 - 39.83	23.72 - 40.2
Lignin	24 - 58	7.74 - 16	7.72 – 12.2
Cellulose	6.8 - 29.5	15.47	8.9 - 14.28
Hemicellulose	4.2 - 22.3	13.63	4.42 - 11.14

[1] (Leite et al., 2016), [2](Salgado et al., 2014a), [3] (Manzanares et al., 2020), [4] (Martínez-Avila et al., 2021), [5] (Chebaibi et al., 2019), [6] (Antónia Nunes et al., 2018) [7] (Sousa et al., 2022), [8] (Shi et al., 2015), [9] (Gao et al., 2023), [10] (Egües et al., 2010), [11] (Martin et al., 2019) [12] (Balan et al., 2009), [13] (de Castro et al., 2016), [14] (Petraru et al., 2021)

The conventional strategies usually employed for oil cakes valorization involves their utilization as animal feed or for energy production (Ancuţa and Sonia, 2020). By-products from rapeseed and sunflower oil extraction are mainly used in animal feed since these materials are characterized by a high content of protein (Lomascolo et al., 2012). However, the presence of some anti-nutritional compounds can reduce their nutritional properties, resulting in a negative impact on animal health and growth performance. In particular, RC and SC contain phytic acid and trypsin inhibitors, which can negatively affect protein digestibility (Ancuţa and Sonia, 2020). Moreover, RC is also characterized by the presence of glucosinolates, which are hydrolyzed during crushing or after ingestion, releasing toxic compounds that led to, for instance, a reduction in feed intake and animal growth (Tripathi and Mishra, 2007).

Although some studies reported the use of OC in animal feed formulations (Abid et al., 2020; Neofytou et al., 2020), the low protein content and, in contrast, the high percentage of fiber and phenolic compounds limits their application in feed industries. Nowadays, after two-phase olive oil extraction, OC is submitted to an additional oil extraction process that is performed, firstly, by malaxation and then by solvent extraction. Before the extraction with solvents, the water content in OC must be reduced to 6 – 8% through a drying process, which is highly energy demanding. After removing the solvent from the remaining solids, dried exhausted OC is mainly used for energy production (Peri, 2014). Besides the energy used for OC drying, burning these materials for energy purposes is not in accordance with the European Parliament Directive 2008/98/EC, which states that waste management should be performed without endanger the environment or human health (EUR-Lex, 2008). Moreover, in 2021, the production of olive oil in Portugal reached 229 million liters, the highest value ever recorded (Instituto Nacional de Estatística, 2022). Ultimately, the OC processing units were unable to process the high volume of OC, which is produced in a short period of time, and this led to suspension of olive harvesting.

As stated above, vegetable oil extraction results in the production of high volumes of oil cakes and the current strategies used for their reuse are associated with some drawbacks. Thus, exploitation of alternative valorization strategies to increase the nutritional properties and economical value of RC and SC should be investigated. Regarding OC, although several studies reported the recovery of added value compounds from olive by-products and their application in food industry (reviewed in Gullón et al. (2020)), this oil cake is still underexplored and other approaches should be further examined.

2.3 VALORIZATION OF OIL CAKES USING BIOTECHNOLOGICAL APPROACHES

Numerous added-value compounds with industrial interest are present in oil cakes and their extraction constitutes a valorization strategy of these materials. Since RC and SC are rich in protein (Table 2.1), several methodologies can be employed for oil cakes' protein extraction (Arrutia et al., 2020). The extracted proteins have potential applications in human nutrition, thus reducing the dependence on animal derived protein, as well as in pharmaceuticals and biodegradable films production (Kaur and Ghoshal, 2022; Wanasundara, 2011). Although OC is not characterized by a high content of protein, other industrially relevant biocompounds can be extracted from this by-product, including hydroxytyrosol, maslinic acid and oleanolic acid, compounds with anti-inflammatory, antioxidant and antimicrobial activities (Xie et al., 2019) and lignin, a biopolymer with high concentrations in OC (Table 2.1) with applications in bioplastic, biofuels and food industries (Cequier et al., 2019). Moreover, other biotechnological approaches can take advantage of the oil cakes chemical composition for the production of industrially relevant value-added compounds by microorganisms.

2.3.1 Solid state and submerged fermentations

Another alternative option for valorization of oil cakes involves their utilization in biotechnological processes, including solid state fermentation (SSF) and submerged fermentation (SmF). SSF is a biotechnological process involving the growth of microorganisms in a solid substrate in the absence or nearly absence of free water in the system (Lizardi-Jiménez & Hernández-Martínez, 2017; Ramos-Sánchez et al., 2015). Moreover, the direct utilization of by-products resultant from agro-industrial activities, such as oil cakes, allows a reduction in the raw material costs, making the production processes economically attractive (Manan & Webb, 2017; Pessôa et al., 2019). Filamentous fungi are widely used in SSF since the substrate mimics their natural habitat and these microorganisms are able to grow in media with low moisture content. While most studies report the selection of filamentous fungi for this bioprocess, in recent years, yeast and bacteria have also shown promising results in added-value compound production in SSF (Ramos-Sánchez et al., 2015; Soccol et al., 2017).

Conversely, SmF is a biotechnological process that requires a liquid culture medium for microbial growth. For this reason, before application in these processes, solid materials must undergo a pretreatment, resulting in the production of a hydrolysate rich in sugars released from cellulose and hemicellulose, which can then be metabolized by microorganisms and converted into added-value compounds. These pretreatments can include enzymatic hydrolysis to convert carbohydrates into sugar

monomers, however, more severe pretreatments are often required before this step to increase sugar release (Melati et al., 2019). Different categories of pretreatments can be used to alter the structure of these materials and several factors can affect the pretreatment efficacy, including cellulose crystallinity and its degree of polymerization, the presence of lignin and hemicellulose acetylation (Ab Rasid et al., 2021; Wagle et al., 2022). Milling, griding, ultrasound and microwave irradiation are some examples of physical pretreatments. Physico-chemical methods include hydrothermal pretreatments, steam explosion and supercritical carbon dioxide extraction. Moreover, in chemical pretreatments acids, alkalis and organic solvents are selected to modify the lignocellulosic structure of the solid by-products (Wagle et al., 2022). In this category are also included ionic liquids (ILs), which are novel solvents with uprising applications in lignocellulosic biomass pretreatment. Interestingly, combination of different anions and cations during ILs synthesis results in selective solubility towards different fractions of the biomass (Usmani et al., 2020). Before pretreatment selection, the composition of the by-product, the environmental impact of the method employed and the overall costs of the pretreatment should be analyzed.

The selection of SSF over SmF for value-added compound production have several advantages (Figure 2.1). While SmF requires constant medium agitation to ensure proper microbial growth and metabolites production, in SSF substrate agitation is very reduced or, in some cases, even absent. Likewise, energy requirements in SSF are reduced and, since agro-industrial by-products can be selected as substrates, this biotechnological process can be performed at lower costs and is considered an eco-friendly alternative compared to SmF. The fact that lower volumes of water are required for this biotechnological process results in a reduction of wastewater production and, on the other hand, allows the obtention of value-added compounds with higher titers, facilitating the downstream purification processes (Abdul Manan and Webb, 2017; Leite et al., 2021). The advantages and disadvantages of SSF with respect to SmF are summarized in Figure 2.1 (Leite et al., 2021).

The chemical composition of OC, SC and RC makes these by-products from vegetable oil industries suitable substrates to be employed in SSF and SmF. In the next sections, the utilization of these oil cakes as substrates in these biotechnological processes is discussed.

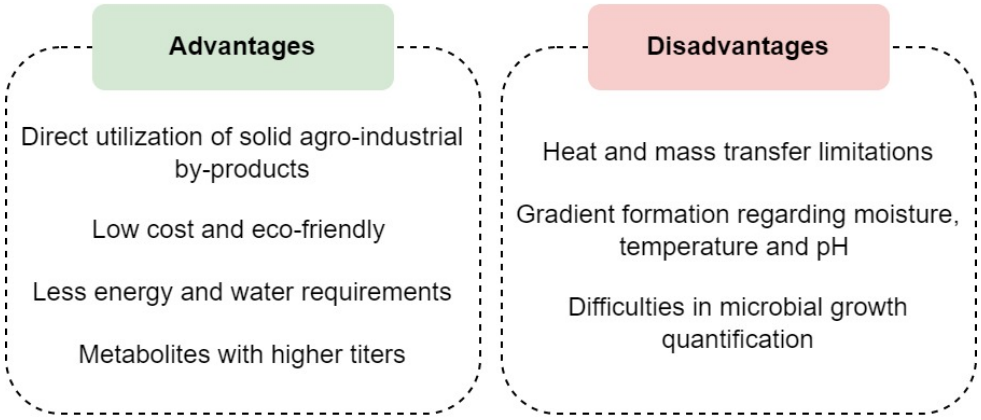

Figure 2.1: Advantages and disadvantages of SSF compared to SmF.

2.3.1.1 Oil cakes from rapeseed and sunflower oil extraction

RC and SC have been previously proposed as substrates in SSF processes for lignocellulosic enzyme production, since the high content of fibers in these materials induces the production of these enzymes (Sousa et al., 2023, 2022). Moreover, the high content of nitrogen in these oil cakes results in a balanced C/N ratio, reducing the need for substrate supplementation to improve microbial growth. Moreover, SSF of RC and SC has also been used for substrate biotransformation and improvement of the nutritional value of these oil cakes. Sousa et al. (2022) reported that SSF of SC and RC with *Aspergillus niger, Aspergillus ibericus* and *Aspergillus oryzae* resulted in a decrease in fiber content and an increase in antioxidant activity compared to the unfermented oil cakes. Similarly, SSF of a mixture of RC (70%) and wheat bran (30%) with *A. niger* led to a decrease in glucosinolates, phytic acid and neutral detergent fiber (NDF) and improved the content of crude protein (Shi et al., 2015). Inclusion of fermented RC in feed formulations had a positive effect on the performance of broiler chickens (Konkol et al., 2023) and improved fatty acid profile of milk produced by dairy cows (Gao et al., 2023). Moreover, SSF with *A. niger* improved the nutritional value of SC and allowed a higher inclusion of the fermented oil cake in shrimp diets in comparison to the unfermented by-product (Jannathulla et al., 2018). The authors also reported that the replacement of fishmeal with the fermented plant protein could reduce the overall cost of feed formulations. The results from these studies revealed that SSF with RC and SC can promote an improvement in the nutritional properties of the fermented oil cakes, which were successfully incorporated in feed formulations, having a positive effect on animal's performance and the quality of their products.

The pretreatment of these oil cakes and the use of hydrolysates in SmF is not frequently reported. Conversely, some studies described the pretreatment of other by-products from rapeseed and sunflower processing, such as rapeseed straw (Rozenfelde et al., 2021) and sunflower stalk (Nargotra et al., 2018), for the obtention of high concentration of sugars and other metabolites. Moreover, the production of xylitol (López-Linares et al., 2018; Martínez et al., 2012) and ethanol (Martínez et al., 2012) using rapeseed and sunflower by-products hydrolysates has also been reported.

2.3.1.2 Oil cakes from olive oil extraction

As stated above, the chemical composition of OC presents significant differences compared to the other oil cakes, which constitutes a challenge for the use of OC in biotechnological processes. Despite this fact, this by-product has been selected as the substrate for SSF and SmF. As can be observed in Table 2.3, OC has been mostly used in fermentations in solid state for enzyme production. Nonetheless, production of astaxanthin by the yeasts *Xanthophyllomyces dendrorhous* and *Sporidiobolus salmonicolor* (Eryilmaz et al., 2016) and biosurfactants by *Bacillus subtilis* (Zouari et al., 2014) in SSF with olive mill by-products have been reported. While some studies described the use of OC as the sole component of the substrate in SSF (Eryilmaz et al., 2016; Leite et al., 2016; Moftah et al., 2012), most of the reports combined OC with other agro-industrial by-products. Substrate mixture is widely used in SSF since agro-industrial by-products may not have the suitable chemical composition to allow microbial growth and biocompound production. Mixture of OC with wheat bran was performed in SSF with *Aspergillus* spp. (Oliveira et al., 2016) and *Y. lipolytica* IMUFRJ 50682 (Lopes et al., 2016) for lipase production. This strategy resulted in a more balanced C/N ratio, improving the chemical composition of the SSF substrate and allowing higher microbial growth and enzyme production. While nitrogen supplementation can still be necessary to improve enzyme production (Lopes et al., 2016; Salgado et al., 2014b), the mixture of OC with other by-products can reduce the need for this synthetic supplementation, lowering the overall costs of biocompound production. Mixture of OC with grape marc (Salgado et al., 2014b), vine trimming shoots and exhausted grape marc (Filipe et al., 2020) were the strategies employed for lipase and lignocellulosic enzymes production by *Aspergillus* spp., respectively. Moreover, *Trichoderma asperellum* was selected for SSF with a mixture of OC, vine shoots and jatropha cake supplemented with olive oil for the production of several fungal enzymes, the lactone 6-pentyl-α-pyrone and conidia (Rayhane et al., 2019). In the same study, increase of scale of this SSF process was successfully performed using 9 kg of the substrate mixture.

Table 2.2: Olive cake application in biotechnological processes for biocompound production.

Process	Products	Microorganism	Additional information	Reference
SSF	Astaxanthin	*Xanthophyllomyces dendrorhous* and *Sporidiobolus salmonicolor*	-	(Eryilmaz et al., 2016)
	Lipase and protease	*Candida utilis*	Alkaline pretreatment	(Moftah et al., 2012)
	Surfactants	*Bacillus subtilis*	Mixture with olive leaf residues	(Zouari et al., 2014)
	Lipase	*Aspergillus* spp.	Mixture with wheat bran	(Oliveira et al., 2016)
			Mixture with exhausted grape marc and urea supplementation	(Salgado et al., 2014b)
		Yarrowia lipolytica	Mixture with wheat bran and ammonium sulphate supplementation	(Lopes et al., 2016)
			Alkaline pretreatment	(Moftah et al., 2013)
	Lignocellulosic enzymes	*Aspergillus niger* and *Aspergillus ibericus*	Mixture with vine trimming shoots and exhausted grape marc	(Filipe et al., 2020)
		Aspergillus niger	Acid and ultrasonic pretreatments	(Leite et al., 2016)
	Enzymes, 6-pentyl-α-pyrone and conidia	*Trichoderma asperellum*	Mixture with vine shoots, jatropha cake and olive oil	(Rayhane et al., 2019)
	Animal feed	*Fusarium flocciferum, Beauveria bassiana, Rhizodiscina cf. Lignyota* and *A. niger*	Supplementation with glucose, yeast extract or salt solution	(Chebaibi et al., 2019)
SmF	Ethanol	*Saccharomyces cerevisiae*	Enzymatic hydrolysis	(Georgieva and Ahring, 2007)
		Saccharomyces cerevisiae and *Thermoanaerobacter mathranii*	Wet oxidation and enzymatic hydrolysis	(Haagensen et al., 2009)
		Escherichia coli	Acid pretreatment	(El Asli and Qatibi, 2009)
	Xylitol	*Candida boidinii*	Acid pretreatment	(López-Linares et al., 2020)

Another strategy to improve microbial growth and value-added compound production is the application of pretreatments to the solid substrates before SSF. Leite et al. (2016) observed that SSF with exhausted OC resulted in higher cellulase and xylanase production by *A. niger* compared to crude OC. The authors concluded that the extraction of the residual oil could have functioned as a pretreatment, improving the substrate accessibility and enzyme production. Moreover, xylanase activity was further improved after ultrasound pretreatment of the exhausted OC before SSF. Alkaline pretreatment of OC was also performed before SSF with the yeasts *Y. lipolytica* (Moftah et al., 2013) and *Candida utilis* (Moftah et al., 2012) and increased enzyme production was observed in both studies. The authors hypothesized that this pretreatment could facilitate microbial access to the substrate due to the swelling and disruption of cell wall, which resulted in higher enzyme production. Although most of the studies using OC had the goal to produce biomolecules with industrial interest, some recent reports evaluated the effect of fungal enzymatic activities in the nutritional properties of this by-product. Chebaibi et al. (2019) observed an enrichment in crude protein and a reduction in phenolic compounds in OC after SSF with several filamentous fungi. Additionally, incorporation of OC pretreated with cellulases and xylanases in the feed of growing lambs resulted in higher fiber digestibility and growth performance in comparison to the incorporation of untreated OC (Abid et al., 2020).

Besides SSF, OC has also been employed as culture medium in SmF processes, and as stated above, these solid by-products must be pretreated originating a hydrolysate with high sugar content. Enzymatic hydrolysis with an enzymatic cocktail resulted in 27% of OC saccharification (in terms of glucose yield) and these sugars, together with the free sugars naturally present in OC, where effectively converted into ethanol by *S. cerevisiae* (Georgieva and Ahring, 2007). The low glucose yield during enzymatic hydrolysis could be related to the high content of lignin in OC, which can limit the access of enzymes to cellulose, thus, impairing glucose release. For this reason, more severe pretreatments are often employed to alter the recalcitrant nature of lignocellulosic biomass, improving the overall sugar release and resulting in a higher fractioning and valorization of these solid materials (Melati et al., 2019). Wet oxidation pretreatment before enzymatic hydrolysis of OC led to a significant improvement in glucose and xylose yields compared to the sugar release observed when enzymatic hydrolysis was the only pretreatment employed (Haagensen et al., 2009). However, the authors reported that wet oxidation had a negative effect on ethanol production by the yeast *S. cerevisiae* and the bacteria *Thermoanaerobacter mathranii,* possibly due to the formation of inhibitory compounds during pretreatment. These compounds, which can include acetic acid, phenolic compounds and furans, are produced due to sugars degradation during the pretreatment step and an increase in pretreatment severity often results in higher inhibitory

compounds release (Wagle et al., 2022). When the concentration of these products can have a deleterious effect on microbial growth, hydrolysate detoxification must be performed before SmF. Acid pretreatment of OC resulted in the release of inhibitory compounds and hydrolysate detoxification was performed before ethanol (El Asli and Qatibi, 2009) and xylitol (López-Linares et al., 2020) production by *E. coli* and *Candida boidinii,* respectively. Conversely, the selection of more robust strains that are not inhibited by these compounds could reduce the need for detoxification steps and decrease the bioprocesses overall costs. For instance, xylitol production by *Pichia fermentans* was achieved with a non-detoxified hydrolysate obtained from acid pretreatment of olive pits (Narisetty et al., 2021). Despite the presence of high concentrations of acetic acid, this yeast was able to grow in the hydrolysate and the detoxification step was unnecessary for yeast growth and value-added compounds production.

The studies discussed so far show promising results regarding the use of OC in biotechnological processes, either in SSF or SmF. However, OC is still an underexplored by-product that poses a negative environmental impact, thus, more studies are required to fully explore the potential of OC as substrate in SSF and SmF or its incorporation in animal feed formulations.

2.4 *YARROWIA LIPOLYTICA*

Yarrowia lipolytica is a non-conventional yeast naturally found in environments rich in fats and/or proteins, such as dairy products and meat. It is strictly aerobic and grows at temperatures below 34 °C, being considered non-pathogenic. In fact, several studies performed to assess the safety and efficacy of *Y. lipolytica* showed that this yeast is safe when employed as a production host for food or feed (reviewed in Groenewald *et al.,* 2014). Moreover, this oleaginous yeast is a dimorphic microorganism, growing as yeast single cells or filamentous hyphae in response to several environmental conditions, which may include pH, temperature, oxygen concentration, osmotic stress and carbon and nitrogen sources (Timoumi et al., 2018).

In the last two decades, *Y. lipolytica* has gained significant attention, which is confirmed by the increasing number of scientific publications with this non-conventional yeast. The increase interest in this microorganism is likely related with its remarkable ability to convert a wide range of substrates, including hydrophobic ones, such as triacylglycerols and hydrocarbons, into several value-added compounds (Lopes et al., 2022). These biocompounds, with applications in several industries, include lipases (Lopes et al., 2018; Moujehed et al., 2022), aromas (Braga et al., 2012; Kothari et al., 2022), citric acid (Gao

et al., 2022), polyols (Gao et al., 2022; Liu et al., 2018a) and microbial lipids (Lopes et al., 2018; Pereira et al., 2023).

While utilization of wild-type strains is preferred for some applications, metabolic engineering provides valuable tools to expand even further the variety of substrates that *Y. lipolytica* can metabolize and increase the productivity of specific biocompounds. In comparison to *Saccharomyces cerevisiae*, metabolic engineering of non-conventional yeasts comes with a greater challenge related to the limited number of selection markers and stable plasmids that can be used in these microorganisms as well as an inefficient homologous recombination (Löbs et al., 2017). However, in the recent years, efforts have been made to develop new synthetic biology approaches to overcome these problems, which boosted the utilization of *Y. lipolytica* in bioprocesses even further (Larroude et al., 2020, 2018). Metabolic engineering of *Y. lipolytica* strains resulted in high production, for instance, of lipase (Janek et al., 2020), erythritol (Liu et al., 2020) and microbial lipids (Yan et al., 2020). Moreover, construction of strains with the ability to efficiently metabolize xylose (Ledesma-Amaro et al., 2016), sucrose (Mirończuk et al., 2015) and polysaccharides such as starch (Ledesma-Amaro et al., 2015) have also been reported.

Yarrowia lipolytica has been widely studied in SmF but the studies reporting the utilization of this oleaginous yeast in SSF are less abundant. However, as stated above, *Y. lipolytica* is able to growth in the form of mycelium, which is an advantage for the utilization of this yeast in a SSF process since the penetration of the microorganism in the solid substrate is improved and nutrient availability increases, resulting in higher microbial growth and production rates (Farias et al., 2014).

2.4.1 SSF with *Y. lipolytica*

Yarrowia lipolytica has a high lipolytic and proteolytic activities, leading to the production of several metabolites that may result in substrate biotransformation. Volatile compound production by *Y. lipolytica* CBS 2075 was study in a cheese-like model and an increase in compounds that could affect cheese flavor, namely short-chain ketones, sulfides and furans was observed (Sørensen et al., 2011). Furthermore, SSF with *Y. lipolytica* NCYC 2904 altered the volatile and non-volatile profiles of green coffee beans (Lee et al., 2017). In particular, the authors observed an increase in total alcohol and volatile phenol levels and, on the other hand, a decrease in total sugars, citric and succinic acids, free amino acids and total phenolic compounds concentration. Regarding biotransformation of agro-industrial by-products, this oleaginous yeast was able to reduce the lipid content in fish wastes, resulting in an improvement in fish meal quality (Yano et al., 2008). Additionally, SSF of okara, a by-product from

soybean processing, with *Y. lipolytica* NCYC 2904 (Vong et al., 2016) and a co-culture of *Y. lipolytica* NCYC 2904 and *Rhizopus oligosporus* (Vong et al., 2018) led to the production of several volatile compounds and increased okara antioxidant activity. Accordingly, fermentation of solid substrates by *Y. lipolytica* results in flavor and aroma modulation, as well as an improvement in substrates' nutritional value. As mentioned in the previous section, *Y. lipolytica* is able to produce several value-added biocompounds with applications in numerous fields. Table 2.3 summarizes some studies regarding the production of biocompounds by this yeast through SSF.

Fermentations in solid state can be performed with an inert support, however, the impregnation of this material with culture medium is fundamental to attain microbial growth and biocompound production. *Yarrowia lipolytica* W29 was able to successfully growth in luffa sponge impregnated with biotransformation medium and convert ricinoleic acid present in Castor oil into γ-decalactones, a peach-scent aroma with high significance in the aroma industry (Try et al., 2018). However, the requirement for a complex medium in SSF with inert supports is a drawback in these bioprocesses and, on the other hand, the utilization of agro-industrial by-products as solid substrates provides not only support but also the nutrients required for microbial growth, allowing the reuse of these materials and the reduction of production costs (Leite et al., 2021). As can be seen in Table 2.3, lipases are the main bioproduct obtain in SSF with *Y. lipolytica*. These enzymes are involved in several reactions, such as esterification, transesterification and aminolysis of lipids, hydrolysis of triacylglycerols and synthesis of esters. Their versatility in terms of applications, such as in food industry, wastewater treatment, pharmaceuticals, cosmetics, leather processing and biodiesel production, increased the interest in the production of these enzymes (Navvabi et al., 2018; Salihu et al., 2012). Lip2p, which is encoded by the gene *LIP2,* has been extensively studied and is the main extracellular lipase secreted by *Y. lipolytica* (Fickers et al., 2011). However, other lipolytic proteins produced by this nonconventional yeast have been identified and characterized (Fickers et al., 2005; Kumari and Gupta, 2012). Lipase biosynthesis by *Y. lipolytica* depends on a variety of factors such as nitrogen and carbon sources, temperature, pH and the presence of inductors (Gonçalves et al., 2014). In particular, lipase production can be induced in the presence of hydrophobic substrates, such as fatty acids and oils (Fickers et al., 2011).

Table 2.3: Biocompound production by *Y. lipolytica* in SSF.

***Yarrowia* strain**	**Substrate**	**Product**	**Yield***	**Additional Information**	**Reference**
W29	Luffa sponge	γ-Decalactones	185 - 5107 mg/L	Solid support impregnated with 20 g/L Castor oil, 2 g/L Tween 80 and 6.7 g/L YNB	(Try et al., 2018)
NCIM 3589	Palm kernel cake	Lipase	18.58 U/g	Supplementation with glucose and urea	(Imandi et al., 2010)
	Mustard oil cake	Lipase	57.89 U/g	Supplementation with glucose and urea	(Imandi et al., 2013)
	Sugarcane bagasse and wheat bran	Lipase	9.3 U/g	Supplementation with glucose and urea	(Imandi and Garapati, 2007)
IMUFRJ 50682	Cotton seed cake Soybean bran	Lipase	50±1 U/g 46±1 U/g	Soybean bran supplemented with soybean sludge	(Farias et al., 2014)
	Soybean bran	Lipase	16 U/g	Supplementation with soy soap stock	(Rocha da Silva et al., 2019)
	Soybean hulls	Lipase	32 U/g	Supplementation with yeast extract, peptone and soybean oil	(Nascimento et al., 2021)
	Canola cake Soybean meal	Lipase	102 U/g 93 U/g	Soybean meal supplemented with soybean oil	(Souza et al., 2017)
M53[#]	Okara and buckwheat husk (5:2)	Erythritol	143.3 mg/g	Substrate exposed to *M. flavus* followed by erythritol fermentation by *Y. lipolytica*	(Liu et al., 2018b)
M53-S[§]	Peanut press cake,40 % sesame meal, 10 % cooking oil	Erythritol	184.5 mg/g	-	(Liu et al., 2019)

*Units or mg of biocompound produced per gram of dry substrate or per liter

[#] Mutant strain

[§] Engineered strain

Oil cakes are often used in SSF for lipase production due to the high percentage of residual oil in these materials. *Yarrowia lipolytica* NCIM 3589 was selected for lipase production in SSF with palm kernel cake (Imandi et al., 2010a), mustard oil cake (Imandi et al., 2013) and a mixture of sugarcane bagasse and wheat bran (Imandi and Garapati, 2007). Higher lipase activity was attained when the substrate used in SSF was a material resultant from oil extraction and, therefore, with a higher content of oil. Similarly, the residual oil present in cottonseed cake (Farias et al., 2014) and canola cake (Souza et al., 2017) was enough to induce lipase production by *Y. lipolytica* IMUFRJ 50682. In the same studies, soybean bran (Farias et al., 2014) and soybean meal (Souza et al., 2017) were also used as SSF substrates and high lipase production was only achieved after supplementation with soybean sludge and soybean oil, respectively. Other authors have also reported the supplementation of low fat soybean by-products with oils or other by-products with high content of residual oil (Nascimento et al., 2021; Rocha da Silva et al., 2019), showing that oily substrates are also essential to induce lipase production by *Y. lipolytica* in SSF. Besides oil supplementation, other authors reported high lipase production after supplementing the solid substrate with carbon and/or nitrogen sources (Imandi et al., 2013, 2010; Imandi and Garapati, 2007; Lopes et al., 2016). This step could improve the C/N ratio of the SSF substrate, favoring biomolecules production (Gonçalves et al., 2014). However, as stated above, modulation of the C/N ratio with the mixture of by-products with different chemical composition is preferred in comparison to synthetic supplementation of carbon and nitrogen sources.

Erythritol is a four-carbon polyol that can be used as a sugar substitute. Even though bacteria are able to synthesize erythritol, yeasts are considered the most effective microorganism for the production of the biocompound (Carly and Fickers, 2018). Many factors seem to affect the production of erythritol, including osmotic pressure, pH, temperature, carbon and nitrogen sources and the presence of surfactants (Rakicka et al., 2016; Rzechonek et al., 2018; Vieira da Silva et al., 2018). While most studies regarding the production of erythritol by *Y. lipolytica* were performed in SmF (Liu et al., 2020, 2018a; Vieira da Silva et al., 2018), the production of this polyol by *Y. lipolytica* M53, a mutant strain, through SSF has reported for the first time using okara mixed with barley wheat as solid substrates (Liu et al., 2018b). The authors performed a hydrolysis step with *Mucor flavus* to increase free reducing sugars availability before SSF with the oleaginous yeast. Furthermore, pH of the substrate mixture was kept at 4 and supplementation with NaCl was performed to increase osmotic pressure, conditions that are fundamental for erythritol production. The deletion of *SNF1* in *Y. lipolytica* M53 resulted in a strain able to produce higher yields of erythritol (Liu et al., 2020). The engineering strain *Y. lipolytica* M53-S was also

selected for erythritol production through SSF with a mixture of peanut press cake, sesame meal and cooking oil (Liu et al., 2019).

These studies revealed that utilization of *Y. lipolytica* in SSF, either for value-added compound production or substrate biotransformation, is a promising approach for valorization of agro-industrial by-products. However, the number of studies with this oleaginous yeast in SSF is still limited and other aspects of this bioprocess should be taken into account and optimized, for example, the increase of scale and strategies to improve microbial accessibility to the substrate.

2.5 REFERENCES

Ab Rasid, N.S., Shamjuddin, A., Abdul Rahman, A.Z., Amin, N.A.S., 2021. Recent advances in green pre-treatment methods of lignocellulosic biomass for enhanced biofuel production. J. Clean. Prod. 321, 129038. https://doi.org/10.1016/j.jclepro.2021.129038

Abdul Manan, M., Webb, C., 2017. Modern microbial solid state fermentation technology for future biorefineries for the production of added-value products. Biofuel Res. J. 4, 730–740. https://doi.org/10.18331/BRJ2017.4.4.5

Abid, K., Jabri, J., Ammar, H., Ben Said, S., Yaich, H., Malek, A., Rekhis, J., López, S., Kamoun, M., 2020. Effect of treating olive cake with fibrolytic enzymes on feed intake, digestibility and performance in growing lambs. Anim. Feed Sci. Technol. 261. https://doi.org/10.1016/j.anifeedsci.2020.114405

Akay, F., Kazan, A., Celiktas, M.S., Yesil-Celiktas, O., 2015. A holistic engineering approach for utilization of olive pomace. J. Supercrit. Fluids 99, 1–7. https://doi.org/10.1016/j.supflu.2015.01.025

Amaral, C., Lucas, M.S., Coutinho, J., Crespí, A.L., do Rosário Anjos, M., Pais, C., 2008. Microbiological and physicochemical characterization of olive mill wastewaters from a continuous olive mill in Northeastern Portugal. Bioresour. Technol. 99, 7215–7223. https://doi.org/10.1016/j.biortech.2007.12.058

Ancuţa, P., Sonia, A., 2020. Oil press-cakes and meals valorization through circular economy approaches: A review. Appl. Sci. 10, 1–31. https://doi.org/10.3390/app10217432

Antónia Nunes, M., Costa, A.S.G., Bessada, S., Santos, J., Puga, H., Alves, R.C., Freitas, V., Oliveira, M.B.P.P., 2018. Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components. Sci. Total Environ. 644, 229–236. https://doi.org/10.1016/j.scitotenv.2018.06.350

Arrutia, F., Binner, E., Williams, P., Waldron, K.W., 2020. Oilseeds beyond oil: Press cakes and meals supplying global protein requirements. Trends Food Sci. Technol. 100, 88–102. https://doi.org/10.1016/j.tifs.2020.03.044

Azbar, N., Bayram, A., Filibeli, A., Muezzinoglu, A., Sengul, F., Ozer, A., 2004. A review of waste management options in olive oil production. Crit. Rev. Environ. Sci. Technol. 34, 209–247. https://doi.org/10.1080/10643380490279932

Balan, V., Rogers, C.A., Chundawat, S.P.S., Da Costa Sousa, L., Slininger, P.J., Gupta, R., Dale, B.E., 2009. Conversion of extracted oil cake fibers into bioethanol including DDGS, canola, sunflower, sesame, soy, and peanut for integrated biodiesel processing. J. Am. Oil Chem. Soc. 86, 157–165. https://doi.org/10.1007/s11746-008-1329-4

Braga, A., Gomes, N., Belo, I., 2012. Lipase induction in *Yarrowia lipolytica* for castor oil hydrolysis and its effect on γ-decalactone production. J. Am. Oil Chem. Soc. 89, 1041–1047. https://doi.org/10.1007/s11746-011-1987-5

Carly, F., Fickers, P., 2018. Erythritol production by yeasts : a snapshot of current knowledge. Yeast 35,

455–463. https://doi.org/10.1002/yea.3306

Cequier, E., Aguilera, J., Balcells, M., Canela-Garayoa, R., 2019. Extraction and characterization of lignin from olive pomace: a comparison study among ionic liquid, sulfuric acid, and alkaline treatments. Biomass Convers. Biorefinery 9, 241–252. https://doi.org/10.1007/s13399-019-00400-w

Chebaibi, S., Leriche Grandchamp, M., Burgé, G., Clément, T., Allais, F., Laziri, F., 2019. Improvement of protein content and decrease of anti-nutritional factors in olive cake by solid-state fermentation: A way to valorize this industrial by-product in animal feed. J. Biosci. Bioeng. 128, 384–390. https://doi.org/10.1016/j.jbiosc.2019.03.010

Contreras, M. del M., Romero, I., Moya, M., Castro, E., 2020. Olive-derived biomass as a renewable source of value-added products. Process Biochem. 97, 43–56. https://doi.org/10.1016/j.procbio.2020.06.013

de Castro, A.M., Castilho, L. dos R., Freire, D.M.G., 2016. Characterization of babassu, canola, castor seed and sunflower residual cakes for use as raw materials for fermentation processes. Ind. Crops Prod. 83, 140–148. https://doi.org/10.1016/j.indcrop.2015.12.050

Dermeche, S., Nadour, M., Larroche, C., Moulti-mati, F., Michaud, P., 2013. Olive mill wastes : Biochemical characterizations and valorization strategies. Process Biochem. 48, 1532–1552. https://doi.org/10.1016/j.procbio.2013.07.010

Egües, I., Alriols, M.G., Herseczki, Z., Marton, G., Labidi, J., 2010. Hemicelluloses obtaining from rapeseed cake residue generated in the biodiesel production process. J. Ind. Eng. Chem. 16, 293–298. https://doi.org/10.1016/j.jiec.2010.01.036

El Asli, A., Qatibi, A.I., 2009. Ethanol production from olive cake biomass substrate. Biotechnol. Bioprocess Eng. 14, 118–122. https://doi.org/10.1007/s12257-008-0071-y

Eryilmaz, E.B., Dursun, D., Dalgiç, A.C., 2016. Multiple optimization and statistical evaluation of astaxanthin production utilizing olive pomace. Biocatal. Agric. Biotechnol. 7, 224–227. https://doi.org/10.1016/j.bcab.2016.06.012

EUR-Lex, 2008. Directive 2008/98/EC of the European Parliament and of the Council. Off. J. Eur. Union.

European Comission, 2023. Olive oil production [WWW Document]. URL https://agridata.ec.europa.eu/extensions/DashboardOliveOil/OliveOilProduction.html (accessed 6.20.23).

Farias, M.A., Valoni, E.A., Castro, A.M., Coelho, M.A.Z., 2014. Lipase production by *Yarrowia lipolytica* in solid state fermentation using different agro industrial residues. Chem. Eng. Trans. 38, 301–306. https://doi.org/10.3303/CET1438051

Feng, D., Zuo, J., 2015. Nutritional and anti-nutritional composition of rapeseed meal and its utilization as a feed ingredient for animal. Int. Consult. Gr. Res. Rapeseed 265–271.

Fickers, P., Fudalej, F., Le Dall, M.T., Casaregola, S., Gaillardin, C., Thonart, P., Nicaud, J.M., 2005. Identification and characterisation of LIP7 and LIP8 genes encoding two extracellular triacylglycerol lipases in the yeast Yarrowia lipolytica. Fungal Genet. Biol. 42, 264–274.

https://doi.org/10.1016/j.fgb.2004.12.003

Fickers, P., Marty, A., Nicaud, J.M., 2011. The lipases from *Yarrowia lipolytica*: Genetics, production, regulation, biochemical characterization and biotechnological applications. Biotechnol. Adv. 29, 632–644. https://doi.org/10.1016/j.biotechadv.2011.04.005

Filipe, D., Fernandes, H., Castro, C., Peres, H., Oliva-Teles, A., Belo, I., Salgado, J.M., 2020. Improved lignocellulolytic enzyme production and antioxidant extraction using solid-state fermentation of olive pomace mixed with winery waste. Biofuels, Bioprod. Biorefining 14, 78–91. https://doi.org/10.1002/bbb.2073

Gao, M., Cieślak, A., Huang, H., Gogulski, M., Petrič, D., Ruska, D., Patra, A.K., El-Sherbiny, M., Szumacher-Strabel, M., 2023. Effects of raw and fermented rapeseed cake on ruminal fermentation, methane emission, and milk production in lactating dairy cows. Anim. Feed Sci. Technol. 300. https://doi.org/10.1016/j.anifeedsci.2023.115644

Gao, Y., Wang, F., Li, X., Mao, G., Xie, H., Song, A., Santos, J.C. dos, Zhang, Z., 2022. Tailored production of citric acid and mannitol by Yarrowia lipolytica from corn stover pretreated by glycerol-assisted instant catapult steam explosion. Ind. Crops Prod. 189, 115820. https://doi.org/10.1016/j.indcrop.2022.115820

Georgieva, T.I., Ahring, B.K., 2007. Potential of agroindustrial waste from olive oil industry for fuel ethanol production. Biotechnol. J. 2, 1547–1555. https://doi.org/10.1002/biot.200700128

Gonçalves, F.A.G., Colen, G., Takahashi, J.A., 2014. *Yarrowia lipolytica* and Its Multiple Applications in the Biotechnological Industry. Sci. World J. 1–14. https://doi.org/10.1155/2014/476207

Groenewald, M., Boekhout, T., Neuvéglise, C., Gaillardin, C., Van Dijck, P.W.M., 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

Gullón, P., Gullón, B., Astray, G., Carpena, M., Fraga-Corral, M., Prieto, M.A., Simal-Gandara, J., 2020. Valorization of by-products from olive oil industry and added-value applications for innovative functional foods. Food Res. Int. 137. https://doi.org/10.1016/j.foodres.2020.109683

Haagensen, F., Skiadas, I. V., Gavala, H.N., Ahring, B.K., 2009. Pre-treatment and ethanol fermentation potential of olive pulp at different dry matter concentrations. Biomass and Bioenergy 33, 1643–1651. https://doi.org/10.1016/j.biombioe.2009.08.006

Imandi, S.B., Garapati, H.R., 2007. Lipase Production by *Yarrowia lipolytica* NCIM 3589 in Solid State Fermentation Using Mixed Substrate. Res. J. Microbiol. https://doi.org/10.3923/jm.2007.469.474

Imandi, S.B., Karanam, S.K., Garapati, H.R., 2013. Use of Plackett-Burman design for rapid screening of nitrogen and carbon sources for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from mustard oil cake (*Brassica napus*). Brazilian J. Microbiol. 44, 915–921. https://doi.org/10.1590/S1517-83822013005000068

Imandi, S.B., Karanam, S.K., Garapati, H.R., 2010. Optimization of media constituents for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from palm Kernal cake (*Elaeis guineensis*).

Adv. Biosci. Biotechnol. 1, 115–121. https://doi.org/10.4236/abb.2010.12016

Instituto Nacional de Estatística, 2022. Estatísticas Agrícolas - 2021.

Janek, T., Mirończuk, A.M., Rymowicz, W., Dobrowolski, A., 2020. High-yield expression of extracellular lipase from *Yarrowia lipolytica* and its interactions with lipopeptide biosurfactants: A biophysical approach. Arch. Biochem. Biophys. 689. https://doi.org/10.1016/j.abb.2020.108475

Jannathulla, R., Dayal, J.S., Ambasankar, K., Muralidhar, M., 2018. Effect of Aspergillus niger fermented soybean meal and sunflower oil cake on growth, carcass composition and haemolymph indices in Penaeus vannamei Boone, 1931. Aquaculture 486, 1–8. https://doi.org/10.1016/j.aquaculture.2017.12.005

Jaswanth, A., Srinivasa Rao, P., Srinivasa Reddy, N., Moulana, M.J., Azeez, M.R., 2022. A Review on Biodiesel Production Technologies. AIP Conf. Proc. 2648. https://doi.org/10.1063/5.0115099

Kaur, R.P., Ghoshal, G., 2022. Sunflower protein isolates-composition, extraction and functional properties. Adv. Colloid Interface Sci. 306, 102725. https://doi.org/10.1016/j.cis.2022.102725

Konkol, D., Jonuzi, E., Popiela, E., Korzeniowska, M., 2023. Influence of solid state fermentation with Bacillus subtilis 67 strain on the nutritional value of rapeseed meal and its effects on performance and meat quality of broiler chickens. Poult. Sci. 102742. https://doi.org/10.1016/j.psj.2023.102742

Kothari, S.D., Vadgama, R.N., Bhat, K.H., Lali, A.M., Odaneth, A.A., 2022. Process optimization for production and purification of γ-decalactone from ricinoleic acid using *Yarrowia lipolytica* NCIM 3590. Biocatal. Agric. Biotechnol. 39, 102285. https://doi.org/10.1016/j.bcab.2022.102285

Kumari, A., Gupta, R., 2012. Extracellular expression and characterization of thermostable lipases, LIP8, LIP14 and LIP18, from *Yarrowia lipolytica*. Biotechnol. Lett. 34, 1733–1739. https://doi.org/10.1007/s10529-012-0958-8

Larroude, M., Rossignol, T., Nicaud, J.M., Ledesma-Amaro, R., 2018. Synthetic biology tools for engineering *Yarrowia lipolytica*. Biotechnol. Adv. 36, 2150–2164. https://doi.org/10.1016/j.biotechadv.2018.10.004

Larroude, M., Trabelsi, H., Nicaud, J.M., Rossignol, T., 2020. A set of *Yarrowia lipolytica* CRISPR/Cas9 vectors for exploiting wild-type strain diversity. Biotechnol. Lett. 42, 773–785. https://doi.org/10.1007/s10529-020-02805-4

Ledesma-Amaro, R., Dulermo, T., Nicaud, J.M., 2015. Engineering *Yarrowia lipolytica* to produce biodiesel from raw starch. Biotechnol. Biofuels 8, 1–12. https://doi.org/10.1186/s13068-015-0335-7

Ledesma-Amaro, R., Lazar, Z., Rakicka, M., Guo, Z., Fouchard, F., Coq, A.M.C. Le, Nicaud, J.M., 2016. Metabolic engineering of *Yarrowia lipolytica* to produce chemicals and fuels from xylose. Metab. Eng. 38, 115–124. https://doi.org/10.1016/j.ymben.2016.07.001

Lee, L.W., Tay, G.Y., Cheong, M.W., Curran, P., Yu, B., Liu, S.Q., 2017. Modulation of the volatile and non-volatile profiles of coffee fermented with *Yarrowia lipolytica*: I. Green coffee. LWT - Food Sci.

Technol. 77, 225–232. https://doi.org/10.1016/j.lwt.2017.01.070

Leite, P., Salgado, J.M., Venâncio, A., Domínguez, J.M., Belo, I., 2016. Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. Bioresour. Technol. 214, 737–746. https://doi.org/10.1016/j.biortech.2016.05.028

Leite, P., Sousa, D., Fernandes, H., Ferreira, M., Costa, A.R., Filipe, D., Gonçalves, M., Peres, H., Belo, I., Salgado, J.M., 2021. Recent advances in production of lignocellulolytic enzymes by solid-state fermentation of agro-industrial wastes. Curr. Opin. Green Sustain. Chem. 27, 100407. https://doi.org/10.1016/j.cogsc.2020.100407

Liu, X., Lv, J., Xu, J.J., Xia, J., He, A., Zhang, T., Li, X., Xu, J.J., 2018a. Effects of osmotic pressure and pH on citric acid and erythritol production from waste cooking oil by *Yarrowia lipolytica*. Eng. Life Sci. 18, 344–352. https://doi.org/10.1002/elsc.201700114

Liu, X., Yan, Y., Zhao, P., Song, J., Yu, X., Wang, Z., Xia, J., Wang, X., 2019. Oil crop wastes as substrate candidates for enhancing erythritol production by modified *Yarrowia lipolytica* via one-step solid state fermentation. Bioresour. Technol. 294, 122194. https://doi.org/10.1016/j.biortech.2019.122194

Liu, X., Yu, X., Wang, Z., Xia, J., Yan, Y., Hu, L., Wang, X., Xu, J., He, A., Zhao, P., 2020. Enhanced erythritol production by a Snf1-deficient *Yarrowia lipolytica* strain under nitrogen-enriched fermentation condition. Food Bioprod. Process. 119, 306–316. https://doi.org/10.1016/j.fbp.2019.11.012

Liu, X., Yu, X., Zhang, T., Wang, Z., Xu, Jiaxing, Xia, J., He, A., Yan, Y., Xu, Jiming, 2018b. Novel two-stage solid-state fermentation for erythritol production on okara–buckwheat husk medium. Bioresour. Technol. 266, 439–446. https://doi.org/10.1016/j.biortech.2018.07.009

Lizardi-Jiménez, M.A., Hernández-Martínez, R., 2017. Solid state fermentation (SSF): diversity of applications to valorize waste and biomass. 3 Biotech 7. https://doi.org/10.1007/s13205-017-0692-y

Löbs, A.K., Schwartz, C., Wheeldon, I., 2017. Genome and metabolic engineering in non-conventional yeasts: Current advances and applications. Synth. Syst. Biotechnol. 2, 198–207. https://doi.org/10.1016/j.synbio.2017.08.002

Lomascolo, A., Uzan-Boukhris, E., Sigoillot, J.C., Fine, F., 2012. Rapeseed and sunflower meal: A review on biotechnology status and challenges. Appl. Microbiol. Biotechnol. 95, 1105–1114. https://doi.org/10.1007/s00253-012-4250-6

Lopes, M., Miranda, S.M., Alves, J.M., Pereira, A.S., Belo, I., 2018. Waste Cooking Oils as Feedstock for Lipase and Lipid-Rich Biomass Production. Eur. J. Lipid Sci. Technol. 1–9. https://doi.org/10.1002/ejlt.201800188

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. Crit. Rev. Biotechnol. 42, 163–183. https://doi.org/10.1080/07388551.2021.1931016

Lopes, V.R.O., Farias, M.A., Belo, I.M.P., Coelho, M.A.Z., 2016. Nitrogen sources on TPOMW valorization

through solid state fermentation performed by *Yarrowia lipolytica*. Brazilian J. Chem. Eng. 33, 261–270. https://doi.org/10.1590/0104-6632.20160332s20150146

López-Linares, J.C., Romero, I., Cara, C., Castro, E., Mussatto, S.I., 2018. Xylitol production by *Debaryomyces hansenii* and *Candida guilliermondii* from rapeseed straw hemicellulosic hydrolysate. Bioresour. Technol. 247, 736–743. https://doi.org/10.1016/j.biortech.2017.09.139

López-Linares, J.C., Ruiz, E., Romero, I., Castro, E., Manzanares, P., 2020. Xylitol production from exhausted olive pomace by *Candida boidinii*. Appl. Sci. 10, 1–16. https://doi.org/10.3390/app10196966

Mailer, R.J., 2016. The Oilseeds, in: Wrigley, C., Corke, H., Seetharaman, K., Faubion, J. (Eds.), Encyclopedia of Food Grains. Elsevier, pp. 221–227.

Manzanares, P., Ballesteros, I., Negro, M.J., González, A., Oliva, J.M., Ballesteros, M., 2020. Processing of extracted olive oil pomace residue by hydrothermal or dilute acid pretreatment and enzymatic hydrolysis in a biorefinery context. Renew. Energy 145, 1235–1245. https://doi.org/10.1016/j.renene.2019.06.120

Martin, A., Osen, R., Greiling, A., Karbstein, H.P., Emin, A., 2019. Effect of rapeseed press cake and peel on the extruder response and physical pellet quality in extruded fish feed. Aquaculture 512, 734316. https://doi.org/10.1016/j.aquaculture.2019.734316

Martínez-Avila, O., Llimós, J., Ponsá, S., 2021. Integrated solid-state enzymatic hydrolysis and solid-state fermentation for producing sustainable polyhydroxyalkanoates from low-cost agro-industrial residues. Food Bioprod. Process. 126, 334–344. https://doi.org/10.1016/j.fbp.2021.01.015

Martínez, M.L., Sánchez, S., Bravo, V., 2012. Production of xylitol and ethanol by *Hansenula polymorpha* from hydrolysates of sunflower stalks with phosphoric acid. Ind. Crops Prod. 40, 160–166. https://doi.org/10.1016/j.indcrop.2012.03.001

Melati, R.B., Shimizu, F.L., Oliveira, G., Pagnocca, F.C., de Souza, W., Sant'Anna, C., Brienzo, M., 2019. Key Factors Affecting the Recalcitrance and Conversion Process of Biomass. Bioenergy Res. 12, 1–20. https://doi.org/10.1007/s12155-018-9941-0

Mirończuk, A.M., Rakicka, M., Biegalska, A., Rymowicz, W., Dobrowolski, A., 2015. A two-stage fermentation process of erythritol production by yeast *Y. lipolytica* from molasses and glycerol. Bioresour. Technol. 198, 445–455. https://doi.org/10.1016/j.biortech.2015.09.008

Moftah, O.A.S., Grbavčić, S., Žuža, M., Luković, N., Bezbradica, D., Knežević-Jugović, Z., 2012. Adding value to the oil cake as a waste from oil processing industry: Production of lipase and protease by *Candida utilis* in solid state fermentation. Appl. Biochem. Biotechnol. 166, 348–364. https://doi.org/10.1007/s12010-011-9429-2

Moftah, O.A.S., Grbavcic, S.Z., Moftah, W.A.S., Lukovic, N.D., Prodanovic, O.L., Jakovetic, S.M., Kneževic-Jugovic, Z.D., 2013. Lipase production by *Yarrowia lipolytica* using olive oil processing wastes as substrates. J. Serbian Chem. Soc. 78, 781–794. https://doi.org/10.2298/JSC120905005M

Moujehed, E., Zarai, Z., Khemir, H., Miled, N., Bchir, M.S., Gablin, C., Bessueille, F., Bonhommé, A., Leonard, D., Carrière, F., Aloulou, A., 2022. Cleaner degreasing of sheepskins by the *Yarrowia*

lipolytica LIP2 lipase as a chemical-free alternative in the leather industry. Colloids Surfaces B Biointerfaces 211. https://doi.org/10.1016/j.colsurfb.2021.112292

Nambi, V.E., 2017. Value Addition of Grains using Solid State Fermentation. Nutr. Food Sci. Int. J. 3, 1–6. https://doi.org/10.19080/nfsij.2017.03.555619

Nargotra, P., Sharma, V., Gupta, M., Kour, S., Bajaj, B.K., 2018. Application of ionic liquid and alkali pretreatment for enhancing saccharification of sunflower stalk biomass for potential biofuel-ethanol production. Bioresour. Technol. 267, 560–568. https://doi.org/10.1016/j.biortech.2018.07.070

Narisetty, V., Castro, E., Durgapal, S., Coulon, F., Jacob, S., Kumar, D., Kumar Awasthi, M., Kishore Pant, K., Parameswaran, B., Kumar, V., 2021. High level xylitol production by *Pichia fermentans* using non-detoxified xylose-rich sugarcane bagasse and olive pits hydrolysates. Bioresour. Technol. 342, 126005. https://doi.org/10.1016/j.biortech.2021.126005

Nascimento, F.V. de, de Castro, A.M., Secchi, A.R., Coelho, M.A.Z., 2021. Insights into media supplementation in solid-state fermentation of soybean hulls by *Yarrowia lipolytica*: Impact on lipase production in tray and insulated packed-bed bioreactors. Biochem. Eng. J. 166, 107866. https://doi.org/10.1016/j.bej.2020.107866

Navvabi, A., Razzaghi, M., Fernandes, P., Karami, L., Homaei, A., 2018. Novel lipases discovery specifically from marine organisms for industrial production and practical applications. Process Biochem. 70, 61–70. https://doi.org/10.1016/j.procbio.2018.04.018

Neofytou, M.C., Miltiadou, D., Sfakianaki, E., Constantinou, C., Symeou, S., Sparaggis, D., Hager-Theodorides, A.L., Tzamaloukas, O., 2020. The use of ensiled olive cake in the diets of Friesian cows increases beneficial fatty acids in milk and Halloumi cheese and alters the expression of SREBF1 in adipose tissue. J. Dairy Sci. 103, 8998–9011. https://doi.org/10.3168/jds.2020-18235

OECD-FAO, 2022. Oilseeds and Oilseed Products. OECD-FAO Agric. Outlook 2022 – 2031 127–138. https://doi.org/10.1787/agr_outlook-2018-7-en

OECD-FAO, 2021. Oilseeds and oilseed products, in: Agricultural Outlook 2021-2030.

Oliveira, F., Moreira, C., Salgado, J.M., Abrunhosa, L., Venâncio, A., Belo, I., 2016. Olive pomace valorization by *Aspergillus* species: lipase production using solid-state fermentation. J. Sci. Food Agric. 96, 3583–3589. https://doi.org/10.1002/jsfa.7544

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. Renew. Energy 202, 1470–1478. https://doi.org/10.1016/j.renene.2022.12.012

Peri, C., 2014. The olive oil refining process, in: The Extra-Virgin Olive Oil Handbook. pp. 201–210. https://doi.org/10.1002/9781118460412.ch17

Pessôa, M.G., Vespermann, K.A.C., Paulino, B.N., Barcelos, M.C.S., Pastore, G.M., Molina, G., 2019. Newly isolated microorganisms with potential application in biotechnology. Biotechnol. Adv. 37, 319–339. https://doi.org/10.1016/j.biotechadv.2019.01.007

Petraru, A., Ursachi, F., Amariei, S., 2021. Nutritional characteristics assessment of sunflower seeds, oil and cake. Perspective of using sunflower oilcakes as a functional ingredient. Plants 10. https://doi.org/10.3390/plants10112487

Rakicka, M., Rywińska, A., Cybulski, K., Rymowicz, W., 2016. Enhanced production of erythritol and mannitol by *Yarrowia lipolytica* in media containing surfactants. Brazilian J. Microbiol. 47, 417–423. https://doi.org/10.1016/j.bjm.2016.01.011

Ramachandran, S., Singh, S.K., Larroche, C., Soccol, C.R., Pandey, A., 2007. Oil cakes and their biotechnological applications - A review. Bioresour. Technol. 98, 2000–2009. https://doi.org/10.1016/j.biortech.2006.08.002

Ramos-Sánchez, L.B., Cujilema-Quitio, M.C., Julian-Ricardo, M.C., Cordova, J., Fickers, P., 2015. Fungal Lipase Production by Solid-State Fermentation. J. Bioprocess. Biotech. 5, 105–116. https://doi.org/10.1007/978-1-59259-991-2_10

Rayhane, H., Josiane, M., Gregoria, M., Yiannis, K., Nathalie, D., Ahmed, M., Sevastianos, R., 2019. From flasks to single used bioreactor: Scale-up of solid state fermentation process for metabolites and conidia production by *Trichoderma asperellum*. J. Environ. Manage. 252, 109496. https://doi.org/10.1016/j.jenvman.2019.109496

Rocha da Silva, J., de Souza, C.E.C., Valoni, E., de Castro, A.M., Coelho, M.A.Z., Ribeiro, B.D., Henriques, C.A., Langone, M.A.P., 2019. Biocatalytic esterification of fatty acids using a low-cost fermented solid from solid-state fermentation with *Yarrowia lipolytica*. 3 Biotech 9, 38. https://doi.org/10.1007/s13205-018-1550-2

Rozenfelde, L., Puke, M., Vedernikovs, N., Scherbaka, R., Rapoport, A., 2021. Catalytic treatment of rapeseed straw for enhanced production of furfural and glucose for bioethanol production. Process Biochem. 102, 102–107. https://doi.org/10.1016/j.procbio.2020.12.007

Rzechonek, D.A., Dobrowolski, A., Rymowicz, W., Mirończuk, A.M., 2018. Recent advances in biological production of erythritol. Crit. Rev. Biotechnol. 38, 620–633. https://doi.org/10.1080/07388551.2017.1380598

Salgado, J.M., Abrunhosa, L., Venâncio, A., Domínguez, J.M., Belo, I., 2014a. Screening of winery and olive mill wastes for lignocellulolytic enzyme production from *Aspergillus* species by solid-state fermentation. Biomass Convers. Biorefinery 4, 201–209. https://doi.org/10.1007/s13399-013-0100-8

Salgado, J.M., Abrunhosa, L., Venâncio, A., Domínguez, J.M., Belo, I., 2014b. Integrated use of residues from olive mill and winery for lipase production by solid state fermentation with *Aspergillus* sp. Appl. Biochem. Biotechnol. 172, 1832–1845. https://doi.org/10.1007/s12010-013-0613-4

Salihu, A., Alam, M.Z., AbdulKarim, M.I., Salleh, H.M., 2012. Lipase production: An insight in the utilization of renewable agricultural residues. Resour. Conserv. Recycl. 58, 36–44. https://doi.org/10.1016/j.resconrec.2011.10.007

Savva, S.C., Kafatos, A., 2016. Vegetable Oils: Dietary Importance, 1st ed, Encyclopedia of Food and Health. Elsevier Ltd. https://doi.org/10.1016/B978-0-12-384947-2.00709-1

Shi, C., He, J., Yu, J., Yu, B., Huang, Z., Mao, X., Zheng, P., Chen, D., 2015. Solid state fermentation of rapeseed cake with *Aspergillus niger* for degrading glucosinolates and upgrading nutritional value. J. Anim. Sci. Biotechnol. 6, 1–7. https://doi.org/10.1186/s40104-015-0015-2

Soccol, C.R., Costa, E.S.F., Letti, L.A.J., Karp, S.G., Vandenberghe, L.P. de S., Woiciechowski, A.L., 2017. Recent developments and innovations in solid state fermentation. Biotechnol. Res. Innov. 1, 52–71. https://doi.org/10.1016/j.biori.2017.01.002

Sørensen, L.M., Gori, K., Petersen, M.A., Jespersen, L., Arneborg, N., 2011. Flavour compound production by *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Debaryomyces hansenii* in a cheese-surface model. Int. Dairy J. 21, 970–978. https://doi.org/10.1016/j.idairyj.2011.06.005

Sousa, D., Salgado, J.M., Cambra-López, M., Dias, A., Belo, I., 2023. Biotechnological valorization of oilseed cakes: Substrate optimization by simplex centroid mixture design and scale-up to tray bioreactor. Biofuels, Bioprod. Biorefining 17, 121–134. https://doi.org/10.1002/bbb.2428

Sousa, D., Salgado, J.M., Cambra-López, M., Dias, A.C.P., Belo, I., 2022. Degradation of lignocellulosic matrix of oilseed cakes by solid-state fermentation: fungi screening for enzymes production and antioxidants release. J. Sci. Food Agric. 102, 1550–1560. https://doi.org/10.1002/jsfa.11490

Souza, C., Farias, M.A., Ribeiro, B.D., Coelho, M.A.Z., 2017. Adding Value to Agro-industrial Co-products from Canola and Soybean Oil Extraction Through Lipase Production Using *Yarrowia lipolytica* in Solid-State Fermentation. Waste and Biomass Valorization 8, 1163–1176.

Timoumi, A., Guillouet, S.E., 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

Tripathi, M.K., Mishra, A.S., 2007. Glucosinolates in animal nutrition: A review. Anim. Feed Sci. Technol. 132, 1–27. https://doi.org/10.1016/j.anifeedsci.2006.03.003

Try, S., De-Coninck, J., Voilley, A., Chunhieng, T., Waché, Y., 2018. Solid state fermentation for the production of γ-decalactones by *Yarrowia lipolytica*. Process Biochem. 64, 9–15. https://doi.org/10.1016/j.procbio.2017.10.004

Usmani, Z., Sharma, M., Gupta, P., Karpichev, Y., Gathergood, N., Bhat, R., Gupta, V.K., 2020. Ionic liquid based pretreatment of lignocellulosic biomass for enhanced bioconversion. Bioresour. Technol. 304, 123003. https://doi.org/10.1016/j.biortech.2020.123003

Vieira da Silva, L., Coelho, M.A.Z., Amaral, P.F.F., Fickers, P., 2018. A novel osmotic pressure strategy to improve erythritol production by *Yarrowia lipolytica* from glycerol. Bioprocess Biosyst. Eng. 41, 1883–1886. https://doi.org/10.1007/s00449-018-2001-5

Vong, W.C., Au Yang, K.L.C., 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

Vong, W.C., Hua, X.Y., Liu, S.Q., 2018. Solid-state fermentation with *Rhizopus oligosporus* and *Yarrowia lipolytica* improved nutritional and flavour properties of okara. LWT - Food Sci. Technol. 90, 316–322. https://doi.org/10.1016/j.lwt.2017.12.050

Wagle, Aditi, Angove, M.J., Mahara, A., Wagle, Amrita, Mainali, B., Martins, M., Goldbeck, R., Raj Paudel, S., 2022. Multi-stage pre-treatment of lignocellulosic biomass for multi-product biorefinery: A review. Sustain. Energy Technol. Assessments 49, 101702. https://doi.org/10.1016/j.seta.2021.101702

Wanasundara, J.P.D., 2011. Proteins of *Brassicaceae* oilseeds and their potential as a plant protein source. Crit. Rev. Food Sci. Nutr. 51, 635–677. https://doi.org/10.1080/10408391003749942

Xie, P., Huang, L., Zhang, C., Deng, Y., Wang, X., Cheng, J., 2019. Enhanced extraction of hydroxytyrosol, maslinic acid and oleanolic acid from olive pomace: Process parameters, kinetics and thermodynamics, and greenness assessment. Food Chem. 276, 662–674. https://doi.org/10.1016/j.foodchem.2018.10.079

Yan, F.X., Dong, G.R., Qiang, S., Niu, Y.J., Hu, C.Y., Meng, Y.H., 2020. Overexpression of △12, △15-Desaturases for Enhanced Lipids Synthesis in *Yarrowia lipolytica*. Front. Microbiol. 11, 1–11. https://doi.org/10.3389/fmicb.2020.00289

Yano, Y., Oikawa, H., Satomi, M., 2008. Reduction of lipids in fish meal prepared from fish waste by a yeast *Yarrowia lipolytica*. Int. J. Food Microbiol. 121, 302–307. https://doi.org/10.1016/j.ijfoodmicro.2007.11.012

Zouari, R., Ellouze-Chaabouni, S., Ghribi-Aydi, D., 2014. Optimization of Bacillus subtilis SPB1 Biosurfactant Production Under Solid-state Fermentation Using By-products of a Traditional Olive Mill Factory. Achiev. Life Sci. 8, 162–169. https://doi.org/10.1016/j.als.2015.04.007

3 VALORIZATION OF BY-PRODUCTS FROM VEGETABLE OIL INDUSTRIES: ENZYMES PRODUCTION BY *YARROWIA LIPOLYTICA* THROUGH SOLID STATE FERMENTATION

Vegetable oil extraction generates high amounts of by-products, designated as oil cakes. Since the current strategies employed for oil cakes' reuse are linked with some drawbacks, identification of alternative approaches to decrease the environmental impact and promote a circular economy is of vital importance. In general, these materials are characterized by high fiber content, making them suitable to be employed in SSF. Filamentous fungi have been the microorganisms mostly applied in SSF and yeasts were applied in less extent. In the present Chapter, three by-products from the extraction of olive, sunflower and rapeseed oils were used as solid substrates in SSF for lipase and protease production by *Y. lipolytica* W29. Oil cakes mixtures composition was optimized for the production of each enzyme using a simplex-centroid design of experiments. A 50% (w/w, dry basis) mixture of OC and SC led to the highest lipase production, while a combination of the three oil cakes was most suitable for maximum protease production. Both enzymes were produced at maximum levels in a short period of 48 h. This work demonstrated that enzyme production by *Y. lipolytica* W29 in SSF can be modulated by the different combinations of oil cakes in the substrate mixture. Additionally, the potential of using by-products from vegetable oil industries in SSF processes was also demonstrated, showing alternative strategies for their revalorization.

This chapter is based on the following research article:

Costa, A.R., Salgado, J.M., Lopes, M., Belo, I., 2022. Valorization of by-products from vegetable oil industries: Enzymes production by *Yarrowia lipolytica* through solid state fermentation. Front. Sustain. Food Syst. 6. https://doi.org/10.3389/fsufs.2022.1006467

3.1 INTRODUCTION

Oleaginous crops are highly cultivated worldwide and their production reached 500 million tons between 2018 and 2020. Moreover, an increase in the production is expected, reaching 600 million tons by 2030 (OECD-FAO, 2021b). These crops are mainly used for oil extraction, which can be used for human consumption and biodiesel production (Waseem et al., 2017). The oil extraction generates large amounts of solid by-products, named oil cakes, and the great demand for vegetable oil production in the next decade will certainly result in increased production of these by-products (OECD-FAO, 2019; Singh et al., 2022).

Oil cakes obtained after rapeseed and sunflower oils extraction, two of the most oilseed crops produced, can be employed as animal feed since these by-products are rich in protein (Lomascolo et al., 2012). However, the presence of antinutritional factors, such as tannins, phytic acid and glucosinolates, may have a negative impact in animal health and growth performance. In contrast, olive oil extraction results in the production of OC with low protein content (Leite et al., 2016). These materials are mainly used for energy production by combustion owing to their high calorific value and are often submitted to a drying process and solvent extraction processes that have a negative environmental impact (Christoforou and Fokaides, 2016). Therefore, more eco-friendly options should be explored. OC is a source of value-added compounds and several green techniques have been used for their extraction (Gullón et al., 2020). Though OC is not usually utilized as animal feedstuff due to its low protein and high fiber content, there are some studies attempting their incorporation in animal feed formulations (Abid et al., 2020b; Joven et al., 2014; Neofytou et al., 2020). Regardless of the oil cakes applications, these by-products are still unexploited and other approaches for their up-grading must be evaluated. Due to their composition, oil cakes can act as a substrate in SSF processes for their biotransformation and the production of bioactive compounds, such as enzymes. However, single oil cakes may not have the suitable nutritional composition to allow optimal microbial growth and target compounds production. Thus, mixing oil cakes from different sources may lead to optimal composition for SSF.

Lipases, which are enzymes that can be obtained by SSF of oil cakes, are involved in several reactions, including hydrolysis of triacylglycerols and synthesis of esters and have applications in numerous fields, such as in food industry, wastewater treatment and biodiesel production (Navvabi et al., 2018; Salihu et al., 2012). Another type of enzymes with industrial relevance are proteases, which are responsible for the hydrolysis of peptide bonds from proteins and polypeptides. These enzymes are mostly used in the detergent, pharmaceutical and food industries (Razzaq et al., 2019).

Yarrowia lipolytica is a non-conventional yeast found in environments rich in fats and/or proteins, such as dairy products (Gonçalves et al., 2014). This oleaginous yeast is broadly used in biotechnological processes to produce enzymes, surfactants, aromas, erythritol and lipids (Lopes et al., 2022). Most of these studies were performed in SmF, however, it the recent years, a growing interest arose in the application of *Y. lipolytica* in SSF processes. The ability of *Y. lipolytica* to grow under mycelium form constitutes an advantage for SSF since the formation of hyphae can increase substrate colonization and nutrients assimilation, resulting in higher microbial growth and production rates. Lipase has been successfully produced by this yeast using, as solid substrates, palm kernel (Imandi et al., 2010), mustard oil (Imandi et al., 2013), cottonseed (Farias et al., 2014) and canola (Souza et al., 2017) cakes and soybean meal (Souza et al., 2017). In most cases, medium supplementation was needed for lipase production. Although two studies reported the production of lipase using by-products from olive oil extraction (Lopes et al., 2016; Moftah et al., 2013), to our knowledge, RC and SC have not yet been explored as solid substrates for the production of this enzyme by *Y. lipolytica*. Despite some reports of protease production by *Y. lipolytica* in SmF and its application in milk proteins hydrolysis (Dąbrowska et al., 2020) and biopeptide synthesis (Pokora et al., 2017), its production in SSF is still underexplored.

In this Chapter, the potential of using OC, RC and SC as solid substrates for lipase and protease production by *Y. lipolytica* in SSF was evaluated. A simplex centroid mixture design was used to obtain the optimum oil cake mixture for the production of these enzymes. Additionally, characterization of the remaining solid substrate after enzyme extraction was performed to evaluate its potential use in other industries, promoting a circular economy.

3.2 Material and Methods

3.2.1 Microorganism

Yarrowia lipolytica W29 (ATCC 20460) was stored at - 80 °C in 30% (v/v) glycerol solution and revived in YPDA medium (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 20 g/L). For inoculum preparation, yeast cells from an agar plate were cultivated overnight in 100 mL of YPD (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L) medium in 500 mL Erlenmeyer flasks in an orbital incubator at 200 rpm and 27 °C.

3.2.2 Oil cakes characterization

RC and SC, acquired in dry conditions from a Portuguese vegetable oil production industry (Iberol SA), were milled and stored at room temperature. OC, collected in wet conditions after olive oil extraction in a two-phase olive mill of the Portuguese northern region (Achsula SA), was stored at -18 °C owing to its high moisture content.

Moisture content and ashes were determined by drying the oil cakes at 105 °C for 24 h and 550 °C for 2 h, respectively, according to the standard methods from AOAC (2005). The quantification of lipids was performed gravimetrically after extraction with chloroform and methanol (2:1, v/v) as described by Ferreira et al. (2020). Total nitrogen was measured by the Kjeldahl method, which was converted to crude protein content by using a factor of 6.25. Organic carbon was quantified using a Thermo Finnigan Flash Element Analyzer 1112 (San Jose, CA USA). Quantification of cellulose, hemicellulose and lignin was performed by quantitative acid hydrolysis as described by Leite et al. (2016). Acid detergent fiber (ADF) was calculated using the percentages of cellulose and lignin. The values of hemicellulose, lignin, and cellulose were used to determine neutral detergent fiber (NDF). To quantify soluble protein, total phenols and reducing sugars, an aqueous extract was obtained by mixing the oil cakes with distilled water at a dry solid/liquid ratio of 1:8 (g:mL) followed by a 30 min incubation at room temperature and 200 rpm. Solid and liquid fractions were separated by filtration with a fine-mesh net and the aqueous extract was centrifuged at 8000 rpm for 10 min and the supernatant was stored at -18 °C.

3.2.3 Optimization of substrate composition for enzymes production under SSF

A simplex centroid mixture design was used to select the best mixture of oil cakes for lipase and protease production by *Y. lipolytica* W29. The components of the mixture, OC, RC and SC, were studied in four levels: 0%, 33.3%, 50% and 100% (w/w) and 9 runs were performed with 2 replicates of the central point. The proportions of oil cakes in each run are presented in Table 3.2.

The following equation is a representation of the model that best fitted the experimental results:

$$Y = \beta 1.x1 + \beta 2.x2 + \beta 3.x3 + \beta 12.x1\,x2 + \beta 13.x1\,x3 + \beta 23.x2\,x3 + \beta 123.x1\,x2\,x3 \quad (1)$$

Where Y represents the predicted response, β is the regression coefficient of the model and x is the independent variables.

Fermentations were carried in 500 mL Erlenmeyer flasks containing 10 g (dry basis) of substrate. Distilled water was added to moist the substrates prior to sterilization at 121 °C for 15 min. The solid substrate was inoculated with 2 mL (3.8 mg of cells per g of solid substrate) of inoculum suspension leading to a moisture content of 75 % (w/w, wet basis) and incubated at 27 °C for 2 days.

Using the best substrate composition, fermentations were performed to evaluate the enzymes production over time by sampling all fermented material in one flask each day. pH in the fermented substrate mixture was determined after sampling (Mettler Toledo LE427). Autoclaved solid substrate without inoculation was used as control and was extracted as described for SSF experiments. Characterization of the control and fermented solid substrates in terms of lipids, fibers and crude protein was performed as described for oil cakes characterization.

3.2.4 Enzymes extraction

For enzymes extraction, a solution of 10 g/L NaCl and 5 g/L Triton X-100 was added to the fermented solid substrate in a dry solid/liquid ratio of 1:8 (g:mL). After 30 min of agitation in an orbital incubator at 200 rpm and room temperature, a liquid extract was obtained by filtration using a fine-mesh net and a sample of the liquid extract was collected for cell counting. This step was followed by centrifugation at 8000 rpm and 4 °C for 10 min. The supernatant was stored at - 18 °C and the remaining solid was dried and stored at room temperature for further analysis.

3.2.5 Analytical methods

The aqueous extracts were characterized in terms of cellular density, lipase and protease activities, reducing sugars and soluble proteins concentration.

Cellular density was determined by cell counting in an optical microscope. The cell number was converted to dry cell mass per liter using a conversion factor. These values were converted to dry cell mass per gram of dry substrate mixture taking into account the volumes of aqueous extract and dry solid recovered after SSF.

Lipase activity measurement was based on a spectrophotometric assay using 4-nitrophenyl butyrate as substrate. Briefly, the enzymatic extract was incubated with 4-nitrophenyl butyrate in sodium acetate buffer 50 mM (pH 5.6) during 15 min at 37 °C. Afterwards, the reaction was stopped by the addition of acetone and 4-nitrophenol release was measured at 405 nm. One unit of lipase activity (U)

was defined as the amount of enzyme that produced 1 µmol of 4-nitrophenol per minute. Lipase activity was expressed in Units per gram of dry substrate (U/g).

Protease activity was determined with a spectrophotometric method based on the reaction of the enzymatic extract with 5 g/L azocasein (Sigma) in sodium acetate buffer 50 mM (pH 5). After incubation for 40 min at 37 °C, 100 g/L trichloroacetic acid was added followed by centrifugation (3000 rpm, 15 min). Potassium hydroxide 5 M was added to the supernatant and the absorbance was measured at 428 nm. One unit of protease activity was defined as the amount of enzyme that produced an increase of 0.01 of absorbance relatively to the blank per minute. Protease activity was expressed in units per gram of dry substrate (U/g).

Reducing sugars were determined using the dinitrosalicylic (DNS) acid reagent method (Miller, 1959) and glucose was used as a standard. Reducing sugars were expressed in mg of reducing sugar per gram of dry substrate (mg/g).

Soluble protein was determined using the Bradford method (Bradford, 1976) and Bovine serum albumin (BSA) was used as a standard. Protein content was expressed in mg of protein per gram of dry substrate (mg/g). The ratio between lipase activity and soluble protein was used to calculate lipase specific activity, which was expressed in units of lipase activity per mg of soluble protein (U/mg).

Total phenols were quantified using the Folin-Ciocalteau method as described by Sousa et al. (2022) and gallic acid was used as a standard. Total phenols were expressed in mg of phenols per gram of dry substrate (mg/g).

3.2.6 Statistical analysis

The results are presented as mean ± standard deviation (SD) of two independent experiments. The experimental design analysis was performed using the Statgraphics Centurion XVI software. The experimental data were statistically evaluated using GraphPad Prism. Data were subjected to one-way analysis of variance (ANOVA) and Tukey's Test for multiple comparison. Analysis was performed with a confidence level of 95%.

3.3 RESULTS

3.3.1 Oil cakes composition

The selection of a solid substrate for SSF must take into account the target compound that will be produced since the substrate composition can affect their production. In the present Chapter, oil cakes, which have residual lipids in their composition and were generated from olive, rapeseed and sunflower oils extraction were used alone or in combination, leading to different substrate composition. The characterization of each oil cake is presented in Table 3.1. The OC used in this work was obtained in a two-phase extraction system, being mainly composed of olive pulp, skins and water. It also presents a high moisture content, which is related to the use of a two-phase centrifugation process for oil extraction (Dermeche et al., 2013; Gullón et al., 2020). Additionally, this by-product has the highest lipid percentage among the three oil cakes used in this work. While a similar carbon content was detected in OC and RC, the carbon percentage in SC is slightly lower. Moreover, OC has a lower nitrogen percentage than the other oil cakes, resulting in the highest C/N ratio for this oil cake (84 for OC and 7 for SC and RC). Thus, crude protein content in OC is 10-fold lower than the values for SC and RC. Some studies reported that a low C/N ratio is needed for biomass production in *Y. lipolytica* (Hapeta et al., 2020) and for lipase production in filamentous fungi (Lima et al., 2003; Rigo et al., 2009). By contrast, synthesis and accumulation of some biocompounds (e.g. microbial lipids by *Y. lipolytica*) are improved by high C/N ratio (Morin et al., 2011). Thus, C/N ratio is an important parameter to control during the production of target bioactive compounds. This can be achieved by medium supplementation, which can often lead to higher production costs, or by the mixture of agro-industrial by-products with different chemical composition. As reported in the literature (Molina-Alcaide and Yáñez-Ruiz, 2008), OC has a high fiber content, having the highest percentage among the three oil cakes used in this work. Regarding the characterization of the aqueous extract, higher content in reducing sugars can be found in OC, showing that this oil cake presents a higher fraction of free sugars readily available for microorganisms assimilation compared to SC and RC. Similarly, OC is also characterized by the highest content in phenolic compounds. Although these compounds may have a negative impact on microbial growth, *Y. lipolytica* is able to not only grow in OMWW based medium but also metabolize the phenolic compounds present in this by-product (Sarris et al., 2011).

The differences regarding oil cakes' chemical composition described so far may be associated with the fruit or seed that originated the oil cake and the method used for oil extraction (Ramachandran

et al., 2007). Moreover, with the mixture of oil cakes at different proportions, it is possible to modulate substrate composition, which can affect microbial growth and biocompounds production.

Table 3.1: Characterization of OC, RC and SC.

Substrate composition	**Oil cakes**		
(%)	**OC**	**RC**[1]	**SC**
Ashes	3.7 ± 0.2[a]	6.7 ± 0.2[a]	6.60 ± 0.06[a]
Lipids	10 ± 1[a]	2.0 ± 0.3[b]	1.32 ± 0.02[b]
Crude protein	3.8 ± 0.3[a]	40 ± 2[b]	40 ± 1[b]
Carbon	51 ± 1[a]	51 ± 1[a]	46 ± 1[b]
Nitrogen	0.61 ± 0.03[a]	8 ± 1[b]	6.4 ± 0.1[b]
ADF	54.6 ± 0.1[a]	23[b]	18 ± 2[b]
NDF	73 ± 2[a]	37[b]	30 ± 2[b]
Total phenols (mg/g)	7.94 ± 0.02[a]	4.8 ± 0.2[b]	6.4 ± 0.5[c]
Reducing sugars (mg/g)	74 ± 3[a]	9.7 ± 0.5[b]	11 ± 1[b]
Moisture (%)	63 ± 1[a]	15.3 ± 0.3[b]	12.0 ± 0.1[b]

Values represent the mean and SD of two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).

[1] (Sousa et al., 2022)

3.3.2 Substrate composition optimization

The utilization of a simplex centroid design allowed the identification of the optimum substrate mixture to obtain the maximum lipase and protease activities after 2 days of SSF (Table 3.2). Besides the quantification of enzymatic activity, cellular density in the aqueous extracts obtained in each experiment was also measured to estimate the influence of oil cakes in yeast growth. Figure 3.1 shows the cellular density obtained in these experiments as a function of the percentage of OC in the substrate mixture. The lowest cellular density was obtained when OC was used as the single component of the solid substrate. Studies using *Y. lipolytica* (Lopes et al., 2016) and filamentous fungi (Salgado et al., 2014) in SSF showed similar results when using this oil cake as the single solid substrate. In spite of the higher content in phenolic compounds of OC compared to the other oil cakes, this difference may not explain the lower cell growth, which is most related with the low nitrogen percentage in the oil cake (Table 3.1). Thereby,

lowering the percentage of OC in the substrate mixture led to an increase in cellular concentration. This result demonstrates that mixture of oil cakes is a suitable strategy for the revalorization of by-products from olive oil production. The highest cellular density was obtained when SC and RC were used alone or in binary mixtures, showing that these by-products are more suitable for *Y. lipolytica* growth than OC.

Table 3.2: Substrate composition for each run of SSF experiments and results of the dependent variables studied in simplex centroid design.

Runs	Independent variables			Dependent variable	
	Oil cakes (%)			Lipase activity (U/g)	Protease activity (U/g)
	OC x_1	RC x_2	SC x_3		
1	100	0	0	1.5 ± 0.4	ND
2	0	100	0	2.1 ± 0.3	57 ± 1
3	0	0	100	3 ± 1	46 ± 3
4	50	50	0	66.4 ± 0.3	11 ± 4
5	50	0	50	97 ± 4	11 ± 4
6	0	50	50	ND	59 ± 1
7	33.3	33.3	33.3	10.7 ± 0.2	59 ± 1
8	33.3	33.3	33.3	17 ± 1	46.38 ± 0.05
9	33.3	33.3	33.3	8 ± 3	58 ± 5

ND: not detected

It has been reported that the presence of lipids in the culture medium induces lipase production (Gonçalves et al., 2014). However, a reduced lipase activity was detected when OC was used as the single component of the solid substrate since yeast growth was low in these conditions (Figure 3.1). The mixture of OC with the other oil cakes improved lipase activity, especially in binary mixtures. As previously mentioned, this combination improved cell proliferation and the residual oil present in OC led to an increase in lipase activity. Similarly, Farias et al. (2014) observed that residual oil on cottonseed cake was essential to attain high values of lipase production by *Y. lipolytica* IMUFRJ 50682. Despite the high cellular growth attained when SC and RC were used alone or in binary mixtures, low lipase activity was observed in these conditions. This result could be related to the low residual oil in these substrates as

well as the release of proteases to the medium that may lead to other enzymes hydrolysis (Ogrydziak, 2013).

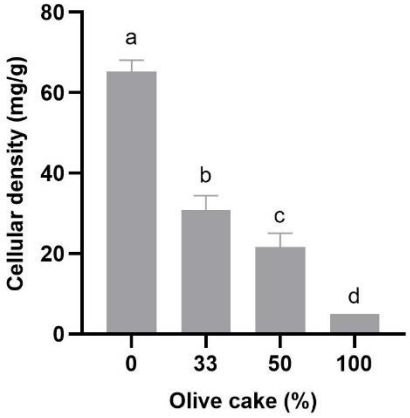

Figure 3.1: Cellular density obtained after two days of SSF as a function of the percentage of olive cake in the substrate mixture. Bars with the same letter are not statistically different ($p > 0.05$).

In fact, *Y. lipolytica* also presents proteolytic activity, being able to synthesize extracellular proteases (Ogrydziak, 2013). The presence of OC alone or in binary mixtures resulted in low protease production. In contrast, high protease activity was detected in the presence of RC and SC in the substrate, either alone, in the binary mixture with these two oil cakes and in ternary mixtures. It has been reported that protease synthesis is induced in protein-rich media (Matkawala et al., 2019; Ogrydziak, 2013) and de Castro et al. (2015) observed a positive correlation between protease production by *A. niger* and the protein content of SSF substrates. Similarly, in this study, protease production by *Y. lipolytica* W29 was improved by the increase of protein content in the solid substrate, that is the case of RC and SC addition to the mixture.

The equations (2) and (3) represent the mathematical models obtained in the simplex centroid mixture design for each enzyme.

$$Lipase = 1.53 * OC + 2{,}09 * SC + 3.03 * SC + 258.32 * OC * RC + 380.48 * OC * SC - 8.36 * RC * SC - 1581.63 * OC * RC * SC \quad (2)$$

$$Protease = 0 * OC + 57.19 * RC + 46.2 * SC - 71.18 * OC * RC - 47.2 * OC * SC + 30.03 * RC * SC + 808.59 * OC * RC * SC \quad (3)$$

The regression coefficients values are directly proportional to the impact that the independent variables have on enzyme activity. Likewise, the behavior of these variables individually or in combination can be examined through these equations. Oil cakes alone had a reduced impact on lipase activity and the use of binary mixtures with OC had a significant positive effect on the production of this enzyme, particularly for the mixture of OC and SC. In contrast, the negative values in the binary mixture with RC and SC and in the ternary mixture revealed an antagonist effect on lipase activity. Regarding the second equation, linear terms had a positive effect, except OC, which had no influence on protease activity. Binary mixture containing OC had an antagonist effect while the binary mixture with SC and RC as well as the ternary mixtures contributed positively for protease activity.

Through variance analysis, a coefficient of determination (R^2) of 0.99 was obtained for lipase activity, which suggests a good fit of the model. Additionally, the *p*-value obtained is lower than 0.05 ($p = 0.0341$), indicating that there is a statistically significant relation, at a 5% level, between lipase activity and the components of the mixture. Concerning protease activity, a high R^2 was also obtained (0.98) and the *p*-value was 0.0635, which revealed that this model is significant at 10% level. Likewise, these results showed that these models can be used for predictive purposes.

The mixture contour plots (Figure 3.2) show the estimated response of lipase and protease activities predicted by the model with the different mixtures of oil cakes. The corners of the triangle represent the components of the mixture and every point inside the triangle corresponds to the enzymes activities predicted with different proportions of oil cakes in the substrate mixture. The regions colored in red represent the substrate composition that led to the maximum enzyme production. According to the plot in Figure 3.2A, highest lipase activity can be achieved when SC and OC are the main components of the mixture. Moreover, the model predicted that lipase activity reaches its maximum, 97 U/g, with a mixture of OC and SC in equal proportions. On the other hand, it appears that highest protease activity is reached with a combination of the three by-products (Figure 3.2B). In particular, a mixture of OC (12%), SC (41%) and RC (47%) led to the optimum protease activity, 64 U/g. Likewise, combination of different agro-industrial by-products with OC appears to be a suitable approach for microbial growth and bioactive compounds production, allowing its revalorization by SSF. Lopes et al. (2016) observed an improvement on lipase activity with the mixture of OC with wheat bran. Similarly, a combination of OC and winery by-products led to higher production of lipase (Salgado et al., 2014) and lignocellulosic enzymes (Filipe et al., 2020). The presence of OC in binary mixtures is essential to attain high lipase activity, while an antagonist effect is observed in these conditions for protease production. Likewise, it appears that the production of lipase and protease can be modulated by the percentage of OC in the solid substrate. The

results obtained in the present work demonstrate that different substrate combinations are required for the optimum production of lipase or protease by *Y. lipolytica*. Thus, changing the substrate composition allows to increase the production of one enzyme over another, facilitating the downstream purification processes. Furthermore, it is possible, at any time, to change the substrate composition to attain the maximum lipase or protease production, depending on by-products availability or market demands, which is in line with the biorefinery concept.

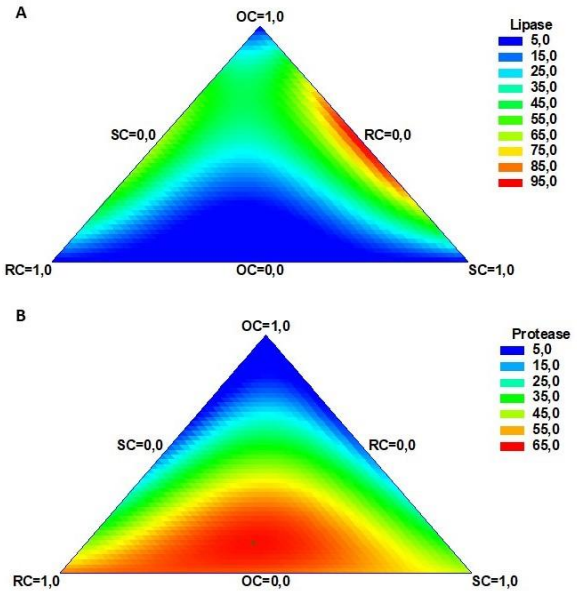

Figure 3.2: Contour plots for the dependent variables obtained in the simplex centroid mixture design. Lipase (A); Protease (B).

3.3.3 Kinetic of SSF

Incubation time is an important parameter to optimize when establishing a SSF process for enzymes production. Hence, after the selection of the best substrate mixture using a standard operation time of two days, SSF of the mixture of 50% (w/w, dry basis) OC and SC was performed for four days to evaluate the profile of lipase and protease production throughout time. A 5-fold increase in cellular concentration was attained after one day of cultivation, reaching 26 mg of cellular dry mass per gram of dry substrate at the end of the experiments (Figure 3.3A). The increase in cellular density was accompanied by a decrease in reducing sugars in the first two days and a residual value was maintained until the end of the SSF process (Figure 3.3A). The total sugars depletion was not observed since *Y.*

lipolytica is unable to efficiently assimilate some monosaccharides, such as xylose, which is included in the hemicellulose fraction of oil cakes. Lipase activity increased in the first day, reaching a maximum of (102 ± 17) U/g at the second day of cultivation (Figure 3.3B), which validates the selection of two days of SSF in the experimental design. Moreover, a lipase specific activity of 3.9 U/mg of total soluble protein was obtained at this point. Lipase production in oily substrates is essential to convert triacylglycerols into free fatty acids that can be incorporated by the cells (Lopes et al., 2022). After being bounded to the yeast cell wall, lipase is gradually released into the medium, increasing the contact between the enzyme and substrate and nutrients assimilation. Lipase secretion begins during the transition to the stationary growth phase when the carbon source is reaching limiting concentrations (Pereira-Meirelles et al., 2000). In this work, maximum lipase activity was observed at the second day of SSF when the cellular growth was already stabilized (Figure 3.3A). Similar lipase activity values were obtained using soybean meal (Souza et al., 2017), a mixture of OC and wheat bran (Lopes et al., 2016) and canola cake (Souza et al., 2017) as solid substrates after 10 h, 24 h and 28 h, respectively, in SSF processes using *Y. lipolytica* IMUFRJ 50682. Moreover, Farias et al. (2014) obtained (46 ± 1) U/g after 14 h of fermentation with *Y. lipolytica* IMUFRJ 50682 using soybean cake supplemented with soybean sludge. In the same work, the potential of cottonseed cake as a SSF substrate was also assessed and, after 28 h, a maximum lipase activity of (50 ± 1) U/g was attained. In contrast, other authors reported longer incubation times to reach maximum enzyme production by *Y. lipolytica*. For instance, four days was the optimum incubation time for lipase production by *Y. lipolytica* NCIM 3589, reaching 18.58 U/g with palm kernel cake as solid substrate (Imandi et al., 2010). The same incubation time was selected in SSF with *Y. lipolytica* NCIM 3589 using mustard oil cake supplemented with glucose and urea (Imandi et al., 2013) and with *Y. lipolytica* NRRL Y-1095 using OC submitted to an alkaline pretreatment (Moftah et al., 2013), leading to a lipase activity of 57.89 U/g and 40 U/g, respectively. In the present work, high values of lipase activity were obtained in a short period of time and without any medium supplementation, which improves enzyme productivity and reduces the production costs of the process. Additionally, these results show the potential of using by-products from olive oil production in combination with SC for lipase production.

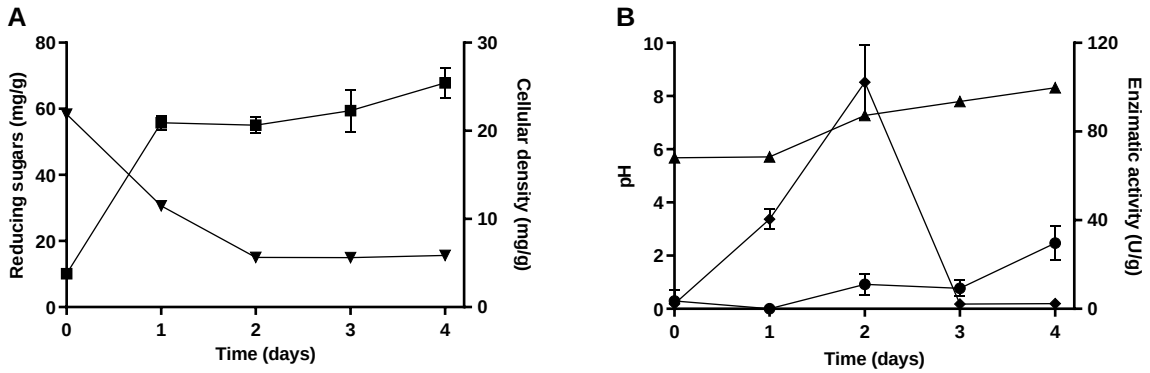

Figure 3.3: Time course of cellular density (■), reducing sugars concentration (▼) (A), enzymatic activity of lipase (♦) and protease (●), and pH (▲) (B) obtained during SSF with *Y. lipolytica* W29 for four days with the optimum substrate mixture for lipase production. The error bars represent the SD of two independent experiments.

After the maximum value of lipase activity was reached, it abruptly decreased until the end of the fermentation. Similar results were obtained during lipase production by *Y. lipolytica* NRRL Y-1095 under SSF using OC (Moftah et al., 2013) and a mixture of OC and wheat bran (Lopes et al., 2016) as solid substrates with *Y. lipolytica* IMUFRJ 50682. To understand the reduction in lipase activity after the second day of SSF, protease activity and pH values were also examined. As can be observed in Figure 3.3B, when lipase activity reached its maximum value, low protease activity was attained. However, in the fourth day of cultivation, when lipase activity was very low, an increase in protease activity was observed. Braga et al. (2012) observed an abrupt reduction in lipase activity, which was followed by an increase in protease production. Moreover, a similar enzymatic activity profile was obtained during SSF using canola cake as the solid substrate (Souza et al., 2017). The increase in pH values and the release of alkaline proteases to the fermentation medium may explain the decrease in lipase production since lipases can be degraded by proteolysis.

The kinetics of protease activity by SSF was also evaluated, with the mixture of the three oil cakes that led to the highest protease production. The yeast cellular density reached was (73 ± 8) mg per gram of dry substrate at the end of the experiments (Figure 3.4A). Despite the lower reducing sugars concentration in this substrate mixture, cellular density was approximately 3-fold higher than that observed in the mixture used at optimal conditions for lipase production. The lower percentage of OC in the optimal substrate mixture for protease production could explain the improved microbial growth, since this substrate mixture is characterized by higher nitrogen percentage. Regardless of the initial

concentration, the final sugars concentration was similar in the two SSF processes. Concerning protease activity, it increased in the first day and (63 ± 3) U/g was obtained after two days of cultivation with the optimized substrate composition (Figure 3.4B), demonstrating that the experimental design executed to predict the protease optimal mixture was correctly performed at two days of SSF. Values of pH also play an important role in protease induction, since *Y. lipolytica* is able to synthesize acid and alkaline proteases, being the latter the most secreted enzyme. As expected, the production of this extracellular protease is induced in a medium with neutral and alkaline pH (Ogrydziak, 2013). Thus, the high protein content in the substrate and the increase of pH values (Figure 3.4B) improved protease activity and the levels of the proteolytic enzyme were maintained until the end of the experiments.

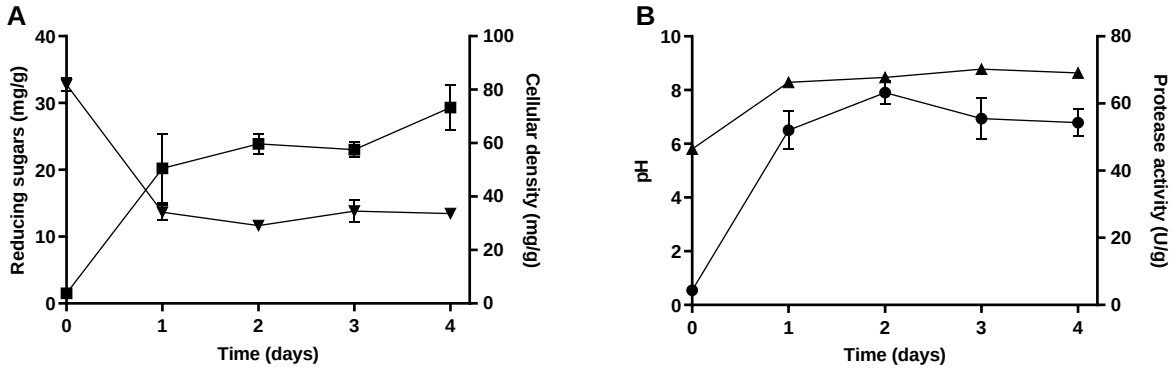

Figure 3.4: Time course of cellular density (■), reducing sugars concentration (▼) (A), protease activity (●), and pH (▲) (B) obtained during SSF with *Y. lipolytica* W29 for four days with the optimum substrate mixture for protease production. The error bars represent the SD of two independent experiments.

Alkaline proteases are produced by *Y. lipolytica* during the exponential growth phase and the production is very reduced or absent in the stationary phase (Ogrydziak, 1993). Unlike lipase, a peak was not observed during protease production possibly because this enzyme was not degraded during the SSF process. To our knowledge, the optimization of SSF processes aiming at protease production by *Y. lipolytica* has not been reported yet. However, as previously mentioned, some reports on lipase production also quantified the proteolytic activity of *Y. lipolytica*. In particular, protease production by *Y. lipolytica* was reported in SSF using canola cake (Souza et al., 2017), soybean by-products (Farias et al., 2014; Souza et al., 2017) and cottonseed cake (Farias et al., 2014) as solid substrates. These results, together with the ones found in the present study, demonstrate the potential of using *Y. lipolytica* as a cell factory for protease production while reusing by-products from vegetable oil industries. Despite the maintenance of

protease activity until the fourth day, since shorter incubation times enhances the process productivity and reduces the production costs, a SSF process of two days can also be selected for protease production by *Y. lipolytica* W29 in these conditions.

3.3.4 Characterization of the fermented substrate

Besides the target biocompounds production, *Y. lipolytica* demonstrated promising results in the biotransformation of agro-industrial by-products, in particular okara (Vong et al., 2018b, 2016), a by-product from soybean processing. Moreover, the high lipolytic and proteolytic activities attained in this work could result in changes in substrate composition. For this reason, after selection of the optimum substrate mixture and incubation time for enzyme production, characterization of the remaining solid after enzyme extraction was performed to evaluate its potential application in other industries or bioprocesses. SSF carried out in the optimal conditions for the production of lipase and protease led to a significant increase in the ash content of the final substrate (Table 3.3). Microbial growth can lead to the loss of dry organic matter such as carbohydrates, lipids, organic acids and proteins. As a result, an apparent increase in the mineral content in the substrate was observed. *Y. lipolytica* growth did not change fiber content, which was an expected outcome since this microorganism is unable to produce lignocellulosic enzymes responsible for the degradation of these materials. In both cases, a slight reduction in crude protein content was observed, which could indicate protein consumption or increased protein solubilization that was removed in the aqueous extract. However, these differences were not statistically significant. Finally, a decrease in lipid percentage was observed, however, these differences were only statistically significant in the optimum substrate mixture for lipase production. This is not surprising since lipase is responsible for the hydrolysis of triacylglycerols and free fatty acids, which are then assimilated by the yeast cells and used as a carbon source (Morin et al., 2011). In the present study, the high lipolytic activity observed in the 50% (w/w, dry basis) mixture of OC and SC could explain the 29% reduction in lipids observed after two days of SSF. Similarly, Yano et al. (2008) reported a reduction in the lipid content of fish mince after SSF with *Y. lipolytica* NBRC-10073. Several studies performed to access the safety of *Y. lipolytica* showed that this microorganism can be used as food or feed (Groenewald et al., 2014) and *Y. lipolytica* biomass has been successfully incorporated in feed formulations (Czech et al., 2016).

Table 3.3: Characterization of the fermented solid substrates after enzymatic extraction.

Parameters (%)	Lipase		Protease	
	Control	Fermented	Control	Fermented
Ashes	4.0 ± 0.4[a]	6.0 ± 0.3[b]	5.0 ± 0.4[a]	10 ± 1[b]
Crude protein	24.4 ± 0.3[a]	21 ± 3[a]	27 ± 2[a]	24 ± 1[a]
NDF	53 ± 5[a]	55 ± 4[a]	43 ± 3[a]	42 ± 3[a]
ADF	38 ± 4[a]	35 ± 4[a]	29 ± 2[a]	29 ± 3[a]
Lipids	6.2 ± 0.2[a]	4.4 ± 0.4[b]	3.54 ± 0.05[a]	2.6 ± 0.5[a]

Values represent the mean and SD of two independent experiments. Values of control and fermented substrate for each enzyme with the same letter are not statistically different ($p > 0.05$).

Accordingly, SSF of the mixture of OC with the other oil cakes enhanced yeast biomass production, which is also a source of protein and can be kept in the solid after SSF if no extraction is performed. The enzymes (lipases and proteases) produced, if also kept in the final fermented mixtures, may have an important role in enhancing nutritional properties of these mixtures by improving lipids and proteins digestibility by the animals (Ojha et al., 2019).

3.4 Conclusions

OC, an underexplored oil cake with very limited use as animal feed, was successfully incorporated in SSF substrate mixtures, opening new opportunities for its valorization. The combination of SC and RC with OC improved the composition of the latter, in particular reduced the C/N ratio, enhancing yeast cellular growth and lipase and protease production in comparison to the utilization of single OC. A simplex centroid mixture design was applied to identify the optimum substrate mixture of OC, RC and SC for maximum lipase and protease production. The content of residual oil in OC was an important factor for lipase production, where 50% (w/w, dry basis) of OC and SC lead to the highest lipase production by SSF. In contrast, high protein content in the SSF substrate, owing to RC and SC, improved protease production. Likewise, the combination of different oil cakes modulated enzyme secretion, resulting in a targeted production. The work present in this Chapter showed the promising application of by-products from vegetable oils production in SSF by *Y. lipolytica*, allowing the production of enzymes of interest for many industries.

3.5 REFERENCES

Abid, K., Jabri, J., Ammar, H., Ben Said, S., Yaich, H., Malek, A., Rekhis, J., López, S., Kamoun, M., 2020. Effect of treating olive cake with fibrolytic enzymes on feed intake, digestibility and performance in growing lambs. Anim. Feed Sci. Technol. 261, 114405. https://doi.org/10.1016/j.anifeedsci.2020.114405

AOAC, 18th editi. ed, 2005. , Official methods of analysis of the Association of Official Analytical Chemists. Washington DC, USA.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 7, 248–254.

Braga, A., Gomes, N., Belo, I., 2012. Lipase induction in *Yarrowia lipolytica* for castor oil hydrolysis and its effect on γ-decalactone production. J. Am. Oil Chem. Soc. 89, 1041–1047. https://doi.org/10.1007/s11746-011-1987-5

Christoforou, E., Fokaides, P.A., 2016. A review of olive mill solid wastes to energy utilization techniques. Waste Manag. 49, 346–363. https://doi.org/10.1016/j.wasman.2016.01.012

Czech, A., Smolczyk, A., Ognik, K., Kiesz, M., 2016. Nutritional value of *Yarrowia lipolytica* yeast and its effect on growth performance indicators n piglets. Ann. Anim. Sci. 16, 1091–1100. https://doi.org/10.1515/aoas-2016-0034

Dąbrowska, A., Bajzert, J., Babij, K., Szołtysik, M., Stefaniak, T., Willak-Janc, E., Chrzanowska, J., 2020. Reduced IgE and IgG antigenic response to milk proteins hydrolysates obtained with the use of non-commercial serine protease from *Yarrowia lipolytica*. Food Chem. 302. https://doi.org/10.1016/j.foodchem.2019.125350

Dermeche, S., Nadour, M., Larroche, C., Moulti-mati, F., Michaud, P., 2013. Olive mill wastes : Biochemical characterizations and valorization strategies. Process Biochem. 48, 1532–1552. https://doi.org/10.1016/j.procbio.2013.07.010

Farias, M.A., Valoni, E.A., Castro, A.M., Coelho, M.A.Z., 2014. Lipase production by *Yarrowia lipolytica* in solid state fermentation using different agro industrial residues. Chem. Eng. Trans. 38, 301–306. https://doi.org/10.3303/CET1438051

Ferreira, M., Fernandes, H., Peres, H., Oliva-Teles, A., Belo, I., Salgado, J.M., 2020. Bio-enrichment of oilseed cakes by *Mortierella alpina* under solid-state fermentation. LWT - Food Sci. Technol. 134. https://doi.org/10.1016/j.lwt.2020.109981

Filipe, D., Fernandes, H., Castro, C., Peres, H., Oliva-Teles, A., Belo, I., Salgado, J.M., 2020. Improved lignocellulolytic enzyme production and antioxidant extraction using solid-state fermentation of olive pomace mixed with winery waste. Biofuels, Bioprod. Biorefining 14, 78–91. https://doi.org/10.1002/bbb.2073

Gonçalves, F.A.G., Colen, G., Takahashi, J.A., 2014. *Yarrowia lipolytica* and Its Multiple Applications in the Biotechnological Industry. Sci. World J. 1–14. https://doi.org/10.1155/2014/476207

Groenewald, M., Boekhout, T., Neuvéglise, C., Gaillardin, C., Van Dijck, P.W.M., 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

Gullón, P., Gullón, B., Astray, G., Carpena, M., Fraga-Corral, M., Prieto, M.A., Simal-Gandara, J., 2020. Valorization of by-products from olive oil industry and added-value applications for innovative functional foods. Food Res. Int. 137. https://doi.org/10.1016/j.foodres.2020.109683

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

Imandi, S.B., Karanam, S.K., Garapati, H.R., 2013. Use of Plackett-Burman design for rapid screening of nitrogen and carbon sources for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from mustard oil cake (Brassica napus). Brazilian J. Microbiol. 44, 915–921. https://doi.org/10.1590/S1517-83822013005000068

Imandi, S.B., Karanam, S.K., Garapati, H.R., 2010. Optimization of media constituents for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from palm Kernal cake (Elaeis guineensis). Adv. Biosci. Biotechnol. 1, 115–121. https://doi.org/10.4236/abb.2010.12016

Joven, M., Pintos, E., Latorre, M.A., Suárez-Belloch, J., Guada, J.A., Fondevila, M., 2014. Effect of replacing barley by increasing levels of olive cake in the diet of finishing pigs: Growth performances, digestibility, carcass, meat and fat quality. Anim. Feed Sci. Technol. 197, 185–193. https://doi.org/10.1016/j.anifeedsci.2014.08.007

Leite, P., Salgado, J.M., Venâncio, A., Domínguez, J.M., Belo, I., 2016. Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. Bioresour. Technol. 214, 737–746. https://doi.org/10.1016/j.biortech.2016.05.028

Lima, V.M.G., Krieger, N., Sarquis, M.I.M., Mitchell, D.A., Ramos, L.P., Fontana, J.D., 2003. Effect of nitrogen and carbon sources on lipase production by *Penicillium aurantiogriseum*. Food Technol. Biotechnol. 41, 105–110.

Lomascolo, A., Uzan-Boukhris, E., Sigoillot, J.C., Fine, F., 2012. Rapeseed and sunflower meal: A review on biotechnology status and challenges. Appl. Microbiol. Biotechnol. 95, 1105–1114. https://doi.org/10.1007/s00253-012-4250-6

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. Crit. Rev. Biotechnol. 42, 163–183. https://doi.org/10.1080/07388551.2021.1931016

Lopes, V.R.O., Farias, M.A., Belo, I.M.P., Coelho, M.A.Z., 2016. Nitrogen sources on TPOMW valorization through solid state fermentation performed by *Yarrowia lipolytica*. Brazilian J. Chem. Eng. 33, 261–270. https://doi.org/10.1590/0104-6632.20160332s20150146

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426–428.

Moftah, O.A.S., Grbavcic, S.Z., Moftah, W.A.S., Lukovic, N.D., Prodanovic, O.L., Jakovetic, S.M., Kneževic-Jugovic, Z.D., 2013. Lipase production by *Yarrowia lipolytica* using olive oil processing wastes as substrates. J. Serbian Chem. Soc. 78, 781–794. https://doi.org/10.2298/JSC120905005M

Molina-Alcaide, E., Yáñez-Ruiz, D.R., 2008. Potential use of olive by-products in ruminant feeding: A review. Anim. Feed Sci. Technol. 147, 247–264. https://doi.org/10.1016/j.anifeedsci.2007.09.021

Morin, N., Cescut, J., Beopoulos, A., Lelandais, G., Le Berre, V., Uribelarrea, J.L., Molina-Jouve, C., Nicaud, J.M., 2011. Transcriptomic analyses during the transition from biomass production to lipid

accumulation in the oleaginous yeast *Yarrowia lipolytica*. PLoS One 6. https://doi.org/10.1371/journal.pone.0027966

Navvabi, A., Razzaghi, M., Fernandes, P., Karami, L., Homaei, A., 2018. Novel lipases discovery specifically from marine organisms for industrial production and practical applications. Process Biochem. 70, 61–70. https://doi.org/10.1016/j.procbio.2018.04.018

Neofytou, M.C., Miltiadou, D., Sfakianaki, E., Constantinou, C., Symeou, S., Sparaggis, D., Hager-Theodorides, A.L., Tzamaloukas, O., 2020. The use of ensiled olive cake in the diets of Friesian cows increases beneficial fatty acids in milk and Halloumi cheese and alters the expression of SREBF1 in adipose tissue. J. Dairy Sci. 103, 8998–9011. https://doi.org/10.3168/jds.2020-18235

OECD-FAO, 2021. OECD-FAO Agricultural Outlook OECD Agriculture statistics (database). https://doi.org/http://dx.doi.org/10.1787/agr-outl-data-en

OECD-FAO, 2019. Oilseeds and oilseed products, in: Agricultural Outlook 2019-2028. pp. 142–153. https://doi.org/10.1787/5f037977-en

Ogrydziak, D., 2013. Acid and Alkaline Extracellular Proteases of *Yarrowia lipolytica*, in: Barth G. (Eds) Yarrowia Lipolytica. Microbiology Monographs. https://doi.org/10.1007/978-3-642-38583-4

Ogrydziak, D.M., 1993. Yeast extracellular proteases. Crit. Rev. Biotechnol. 13, 1–55. https://doi.org/10.3109/07388559309069197

Ojha, B.K., Singh, P.K., Shrivastava, N., 2019. Enzymes in the animal feed industry, in: Enzymes in Food Biotechnology: Production, Applications, and Future Prospects. pp. 93–109. https://doi.org/10.1016/B978-0-12-813280-7.00007-4

Pereira-Meirelles, F. V., Rocha-Leão, M.H.M., Sant'Anna, G.L., 2000. Lipase location in *Yarrowia lipolytica* cells. Biotechnol. Lett. 22, 71–75. https://doi.org/10.1023/A:1005672731818

Pokora, M., Zambrowicz, A., Zabłocka, A., Dąbrowska, A., Szołtysik, M., Babij, K., Eckert, E., Trziszka, T., Chrzanowska, J., 2017. The use of serine protease from *Yarrowia lipolytica* yeast in the production of biopeptides from denatured egg white proteins. Acta Biochim. Pol. 64, 245–253. https://doi.org/10.18388/ABP.2016_1316

Ramachandran, S., Singh, S.K., Larroche, C., Soccol, C.R., Pandey, A., 2007. Oil cakes and their biotechnological applications - A review. Bioresour. Technol. 98, 2000–2009. https://doi.org/10.1016/j.biortech.2006.08.002

Razzaq, A., Shamsi, S., Ali, A., Ali, Q., Sajjad, M., Malik, A., Ashraf, M., 2019. Microbial proteases applications. Front. Bioeng. Biotechnol. 7, 1–20. https://doi.org/10.3389/fbioe.2019.00110

Rigo, E., Ninow, J.L., Polloni, A.E., Remonatto, D., Arbter, F., Vardanega, R., De Oliveira, D., Treichel, H., Di Luccio, M., 2009. Improved lipase biosynthesis by a newly isolated *Penicillium* sp . Ind. Biotechnol. 5, 119–126.

Salgado, J.M., Abrunhosa, L., Venâncio, A., Domínguez, J.M., Belo, I., 2014. Integrated use of residues from olive mill and winery for lipase production by solid state fermentation with *Aspergillus* sp. Appl. Biochem. Biotechnol. 172, 1832–1845. https://doi.org/10.1007/s12010-013-0613-4

Salihu, A., Alam, M.Z., AbdulKarim, M.I., Salleh, H.M., 2012. Lipase production: An insight in the utilization of renewable agricultural residues. Resour. Conserv. Recycl. 58, 36–44.

https://doi.org/10.1016/j.resconrec.2011.10.007

Singh, R., Langyan, S., Sangwan, S., Rohtagi, B., Khandelwal, A., Shrivastava, M., 2022. Protein for Human Consumption From Oilseed Cakes: A Review. Front. Sustain. Food Syst. 6, 1–12. https://doi.org/10.3389/fsufs.2022.856401

Sousa, D., Salgado, J.M., Cambra-López, M., Dias, A.C.P., Belo, I., 2022. Degradation of lignocellulosic matrix of oilseed cakes by solid-state fermentation: fungi screening for enzymes production and antioxidants release. J. Sci. Food Agric. 102, 1550–1560. https://doi.org/10.1002/jsfa.11490

Souza, C., Farias, M.A., Ribeiro, B.D., Coelho, M.A.Z., 2017. Adding Value to Agro-industrial Co-products from Canola and Soybean Oil Extraction Through Lipase Production Using *Yarrowia lipolytica* in Solid-State Fermentation. Waste and Biomass Valorization 8, 1163–1176.

Vong, W.C., Au Yang, K.L.C., 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

Vong, W.C., Hua, X.Y., Liu, S.Q., 2018. Solid-state fermentation with *Rhizopus oligosporus* and *Yarrowia lipolytica* improved nutritional and flavour properties of okara. LWT - Food Sci. Technol. 90, 316–322. https://doi.org/10.1016/j.lwt.2017.12.050

Waseem, S., Imadi, S.R., Gul, A., Ahmad, P., 2017. Oilseed Crops: Present scenario and future prospects, in: Oilseed Crops: Yield and Adaptations under Environmental Stress. pp. 1–306.

Yano, Y., Oikawa, H., Satomi, M., 2008. Reduction of lipids in fish meal prepared from fish waste by a yeast *Yarrowia lipolytica*. Int. J. Food Microbiol. 121, 302–307. https://doi.org/10.1016/j.ijfoodmicro.2007.11.012

4 SOLID STATE AND SEMI-SOLID FERMENTATIONS OF OLIVE AND SUNFLOWER CAKES WITH *YARROWIA LIPOLYTICA*: IMPACT OF BIOLOGICAL AND PHYSICAL PRETREATMENTS

Lignocellulosic biomass is a promising feedstock for value-added compound production in biotechnological processes, such as SSF. Although these solid materials can be directly use as substrates in fermentations in solid state, a pretreatment is often required, especially if the microorganism selected is unable to produce lignocellulosic enzymes. In the present Chapter, several pretreatment strategies were applied to the optimum mixture for lipase production by *Y. lipolytica* W29 before SSF. Biological pretreatment with a fungal enzymatic extract led to a significant increase in sugar availability in the substrate mixture after a short incubation period, improving yeast growth. Microwave and ultrasounds were the physical pretreatments selected and microwave irradiation proved to be the best method, resulting in higher yeast growth and lipase production compared to the untreated mixture. An improvement in lipase activity was also observed after ultrasonic irradiation in semi-solid fermentations. The utilization of pretreatments before SSF with *Y. lipolytica* can increase sugars availability and result in structural changes in the solid substrate, which can improve the bioprocesses' productivity.

The information presented in this chapter was submitted to *Fermentation:*

Costa, A. R.; Fernandes, H.; Salgado, J. M.; Lopes, M.; Belo, I. Solid state and semi-solid fermentations of olive and sunflower cakes with *Yarrowia lipolytica*: impact of biological and physical pretreatments (July 2023).

4.1 INTRODUCTION

Lignocellulosic biomass has been studied and proposed as substrate in biotechnological processes for biocompounds production as an alternative culture medium, making the production process more economically attractive but also contributing to a circular economy, with a positive environmental impact (Leite et al., 2021). These lignocellulosic materials can be directly used as substrate in SSF processes, functioning both as a solid support and a nutrient source (Sadh et al., 2018). In some cases, pretreatment of these materials can be performed to improve substrate accessibility, resulting in higher microbial growth and productivity (Leite et al., 2016; Martínez-Avila et al., 2021; Zhao et al., 2010).

Most of the reports with *Y. lipolytica* in SSF used agro-industrial by-products as the solid substrates with high content of fibers, including by-products from soybean processing (Farias et al., 2014; Liu et al., 2018b; Souza et al., 2017) and olive oil extraction (Lopes et al., 2016). However, since *Y. lipolytica* is unable to produce lignocellulosic enzymes, the cellulose and hemicellulose fractions of these materials are underutilized, and, for this reason, pretreatments before their utilization on biotechnological bioprocesses can be used to overcome this limitation. As an example, alkaline pretreatment of olive by-products prior to SSF resulted in improved lipase and protease production by *C. utilis* (Moftah et al., 2012) and lipase production by *Y. lipolytica* NRRL Y-1095 (Moftah et al., 2013). However, this strategy requires the utilization of harmful chemicals and high amounts of water to neutralize the substrate prior to SSF, thus, other approaches for by-products pretreatment with a lower enviromental impact must be considered. Liu et al., (2018) used a pre-fermented mixture of okara and buckwheat husk by *Mucor flavus* as the SSF substrate for erythritol production by *Y. lipolytica* M53. Similarly, co-culture of *Trichoderma* sp. and *Saccharomyces cerevisiae* in SSF using sweet potato flour as solid substrate resulted in improved bioethanol production by the yeast strain (Swain et al., 2013). Using a different approach, Martínez-Avila et al., (2021) applied fungal enzymatic extracts in enzymatic hydrolysis at high solid loading before SSF for the production of bioplastics by two bacteria strains. The authors from these studies reported that the enzymes produced by the filamentous fungi were fundamental to increase sugar availability in the solid substrate, resulting in improved microbial growth and biocompound production. Besides these biological approaches, physical treatments, such as ultrasound and microwave irradiation, have also been employed in lignocellulosic biomass pretreatment. Ultrasounds pretreatment leads to the formation of microbubbles in the materials and the collapse of these microbubbles can result in structural changes and improve the solubilization of organic matter (Ong and Wu, 2020). On the other hand, microwave pretreatment is a technology that allows to selectively heat the lignocellulosic biomass from the inside out, resulting in increased porosity and surface area (Cantero et al., 2019). Although microwave (Zhao et

al., 2010) and ultrasound (Leite et al., 2016) pretreatments have been used before SSF with filamentous fungi, to our knowledge, the effect of these physical pretreatments on *Y. lipolytica* growth and biocompound production under SSF have not been studied yet.

In Chapter 3, substrate mixture used in SSF for lipase production by *Y. lipolytica* W29 was optimized and maximum activity was attained with a 50% (w/w, dry basis) mixture of OC and SC. This substrate mixture is rich in fibers and, as expected, the microbial growth during the fermentation process did not result in significant changes in the fiber content (Table 3.3). Thereby, in the present Chapter, the effect of physical and biological pretreatments on SSF performance to produce lipases was evaluated (Figure 4.1). Co-culture with filamentous fungi and enzymatic hydrolysis with a fungal enzymatic cocktail were the biological treatments selected as strategies to increase sugar availability in the substrate mixture for SSF. Moreover, microwave and ultrasound pretreatments were also applied to the substrate mixture to evaluate their effect on lipase and biomass production by *Y. lipolytica*.

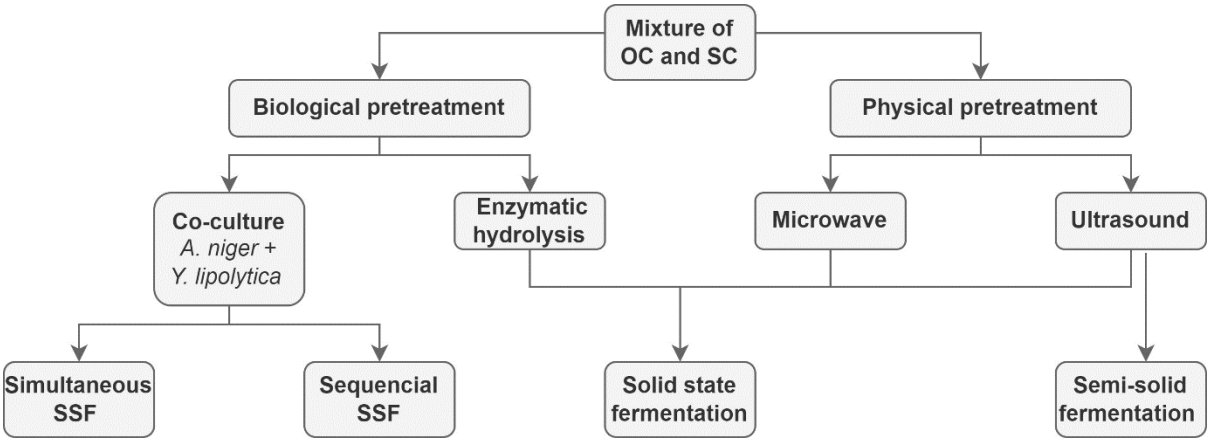

Figure 4.1: Diagram of the pretreatments applied to the mixture of OC and SC.

4.2 MATERIALS AND METHODS

4.2.1 Raw materials

Sunflower, rapeseed and soybean cakes were supplied from a Portuguese vegetable oil production industry (Iberol SA). These materials were obtained in dry conditions and were milled and stored at room temperature. OC were supplied by a two-phase olive mill of the Portuguese northern region (Achsula SA) and was stored at -18 °C due to its high moisture content.

4.2.2 Microorganisms

Yarrowia lipolytica W29 (ATCC 20460) was stored in 30% (v/v) glycerol stocks at -80 °C and revived in YPD agar medium (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 20 g/L). For inoculum preparation, yeast cells were collected from an agar plate and cultivated overnight in 500 mL Erlenmeyer flasks with 100 mL of YPD medium in an orbital incubator at 200 rpm and 27 °C. *Aspergillus niger* CECT 2915 was obtained from CECT (Colección Española de Cultivos Tipo, Valencia, Spain) and preserved at - 80 °C in a glycerol solution. The microorganism was revived in potato dextrose agar (PDA) plates and, prior to substrate inoculation, the spores from a PDA plate were suspended in 1 g/L peptone and 0.1 g/L Tween 80.

4.2.3 Co-culture with *A. niger* and *Y. lipolytica*

Sequential and simultaneously SSF was performed with *A. niger* CECT 2915 and *Y. lipolytica* W29. Fermentations were performed in 500 mL Erlenmeyer flasks with 10 g (dry basis) of 50 % (w/w, dry basis) mixture of OC and SC mixed with distilled water to adjust the moisture content. In the sequential SSF, 2 mL (10^5 spores per gram of solid substrate) of spore suspension of *A. niger* was added to the autoclaved substrate and flasks were kept in an incubator at 27 °C. After two days, 2 mL (3.8 mg of cells per gram of dry solid substrate) of a yeast suspension was added to the fermented mixture, adjusting the final moisture content to 75 % (w/w, wet basis) and flasks were kept at the same temperature for another two days. In SSF with simultaneous inoculation, 2 mL of spore and 2 mL of yeast inoculum suspensions were added to the solid substrate, leading to a final moisture content of 75% (w/w, wet basis). Flasks were kept at 27 °C for two days. A standard SSF assay with a monoculture of *Y. lipolytica* W29 was performed for two days as described in Chapter 3.

4.2.4 Production of enzymatic extract from *A. niger*

The enzymatic extract used in the hydrolysis step was obtained from a SSF process using equal parts of SC, RC and soybean cake as described by Sousa et al. (2023). Flasks were maintained at 25 °C for 7 days. Enzyme extraction was performed by mixing the fermented substrate with 0.05 M citrate buffer (pH 4.8) with a dry solid/liquid ratio of 1:8 (g:mL). After agitation in an orbital incubator for 30 min at room temperature, the liquid extract was recovered by filtration with a fine-mesh net and centrifuged

during 10 min at 8000 rpm and 4 °C. The supernatant was then vacuum filtered with filter paper and stored at -18 °C.

4.2.5 Enzymatic hydrolysis followed by SSF

2 g (dry basis) of the mixture of 50% (w/w, dry basis) of OC and SC was sterilized for 15 min at 121 °C and, after cooling, the crude enzymatic extract was added to this substrate mixture to achieve a final cellulase concentration of 50, 100 and 150 U/g at a solid loading of 25% (w/w, wet basis). A commercial cellulase (Sigma, C1184) in 0.05 M citrate buffer (pH 4.8) was used as a control. Enzymatic hydrolysis was performed at 50 °C and the experiments were followed up to 48 h. After selection of the optimum hydrolysis conditions, 10 g (dry basis) of the optimum substrate mixture for lipase production was autoclaved in 500 mL Erlenmeyer flasks for 15 min at 121 °C and the enzymatic extract was added to the solid materials with a final cellulase concentration of 100 U/g. Flasks were kept in an incubator at 50 °C for 12 h followed by another cycle of sterilization to inactive the enzymes from the crude extract. The substrate mixture was inoculated with 2 mL of yeast cellular suspension, flasks were placed in an incubator at 27 °C and enzyme production over time was evaluated by sampling the whole fermented substrate in one flask each day. Standard SSF assays were performed for two days using water or citrate buffer 0.05 mM (pH 4.8) to adjust the moisture content. Enzyme extraction after SSF was performed as stated in Chapter 3.

4.2.6 Physical pretreatments followed by SSF

Physical pretreatments were performed with 10 g (dry basis) of the substrate mixture in 500 mL Erlenmeyer flasks and distilled water was used to moist the substrate at a solid loading of 25% (w/w, wet basis), similar to biological pretreatment. Flasks were placed in a ultrasounds bath Sonorex Digitec DT 514 (Bandelin, Germany) at 35 kHz for 15 min and, for microwave irradiation, flasks were place in a domestic microwave equipment (1250 W, model MS2387U, LG) at 680 W for 2 min in cycles of 30 seconds. After the pretreatments, substrate sterilization and SSF was performed as described above and enzyme extraction was performed as stated in Chapter 3.

4.2.7 Semi-solid fermentation after ultrasound pretreatment

The mixture of OC and SC was mixed with distilled water at a solid loading of 10% (w/w) and ultrasound pretreatment was performed with an ultrasonic processor Cole-Parmer (Illinois, USA) with 750 W and 20 kHz. The probe of the ultrasonic processor was in contact with the suspension of oil cakes for the required time, which varied from 2 to 15 min. Following sonication, liquid and solid fractions were separated by centrifugation at 8000 rpm for 10 min. The supernatant was stored at - 18 °C for further analysis. After selection of the pretreatment duration, 5 g (dry basis) of the pretreated mixture was transferred to 250 mL Erlenmeyer flasks and sterilized at 121 °C for 15 min. After cooling, 1 mL of yeast inoculum suspension was added to the pretreated mixture and flasks were placed in an orbital incubator at 27 °C and 200 rpm. Liquid samples were collected at specific time points and cell counting was performed to estimate cellular concentration in the suspension. The remaining sample was centrifuged at 8000 rpm for 10 min and the supernatant was stored at - 18 °C for further analysis. Substrate mixture at 10% (w/w) of solid loading without ultrasounds pretreatment was used as a control. After 30 h of fermentation, the semi-solid mixture containing the yeast biomass was dried at 60 °C for 48 h and the dry substrate was stored at room temperature.

4.2.8 Analytical methods

The aqueous extract obtained after SSF was characterized regarding reducing sugars content, cellular concentration and enzymatic activities of protease and lipase as described in Chapter 3.

The activities of cellulase and xylanase after SSF with *A. niger* CECT 2915 were quantified as described by Filipe et al. (2020) using carboxymethylcellulose (CMC) and beechwood xylan as substrates, respectively.

Glucose and xylose concentrations were determined by high-performance liquid chromatography (LC 2060C, Shimadzu, Japan) using an Aminex HPX-87H column (300 mm × 7.8 mm, 8 µm particle size) with temperature set at 60 °C and equipped with RI and UV detectors. 5 mM sulfuric acid, at a flow rate of 0.5 mL/min, was selected as the mobile phase.

Fiber content in the substrate mixture pretreated with the crude enzymatic extract and in the control condition was quantified as described in Chapter 3.

Lipid content in the fermented solid after semi-solid fermentation was determined gravimetrically after extraction with methanol and chloroform (2:1, v/v) as described by Ferreira et al. (2020). Fatty acid profile of the lipids present in the fermented substrate mixture was determined by quantification of fatty

acid methyl esters (FAMEs). Fatty acids were converted into FAMEs by methylation using a solution of methanol and sulfuric acid (85:15, v/v) (Lopes et al., 2018). FAMEs analysis was performed by gas chromatography with a CP-3800 gas chromatograph (Varian Inc., USA) equipped with a flame ionized detector (FID) and TRACSIL TR-WAX capillary column (30 m × 0.25 mm x 0.25 mm, Teknokroma, Spain). The initial temperature of the column was kept at 50 °C for 2 min, followed by an increase of 10 °C /min until reaching 225 °C, temperature that was kept for 10 min. The temperatures of the injector and detector were 220 °C and 250 °C, respectively, and helium was selected was the carrier gas (1 mL/min). Heptadecanoic acid was used as an internal standard and FAMEs standards were used to identify FAMEs in the samples by comparison of the retention times. The ratio between fatty acid concentration (g/L) and the sum of all fatty acids quantified was used to calculate the relative amount of each fatty acid.

4.2.9 Statistical analysis

The results are presented as mean ± standard deviation (SD) of two independent experiments. The experimental data were subjected to *t*-test, one-way analysis of variance (ANOVA) and Tukey's test for multiple comparison using GraphPad Prism. All the analysis were performed with a confidence interval of 95%.

4.3 RESULTS AND DISCUSSION

4.3.1 SSF with *A. niger*

In Chapter 3, optimum substrate composition and incubation time were selected for maximum lipase production by *Y. lipolytica* W29 in SSF. While microbial growth resulted in a reduction in the lipid content of this substrate mixture (Table 3.3), significant changes in fiber percentage were not registered. This outcome is due to *Yarrowia* species' inability to produce lignocellulosic enzymes, thus, complex polysaccharides, such as cellulose and hemicellulose, are not hydrolyzed during SSF.

Likewise, mixed cultures of microorganisms that do not secrete these enzymes and filamentous fungi producers of lignocellulolytic enzymes could be a suitable strategy to increase substrate utilization and microbial growth, resulting in high biocompound yields. In the present work, two co-culture strategies with *Y. lipolytica* W29 and *A. niger* CECT 2915 were studied and the influence of co-culture on enzyme production was evaluated. Nevertheless, before the co-culture experiments, SSF with a monoculture of *A.*

niger CECT 2915 was performed to assess enzyme production on a substrate composed of 50 % (w/w, dry basis) of OC and SC. SSF was followed up to four days and the results are present in Table 4.1.

Aspergillus niger is widely used in SSF for lignocellulosic enzymes production (Filipe et al., 2020; Leite et al., 2016; Moran-Aguilar et al., 2021; Sousa et al., 2022). The utilization of lignocellulosic materials as solid substrates in SSF can induce the production of lignocellulosic enzymes, which results in the conversion of the lignocellulosic matrix into single sugars that are easily metabolized by microorganisms (Leite et al., 2021; Sousa et al., 2022). Furthermore, some studies already reported the use of OC (Filipe et al., 2020; Leite et al., 2016) and SC (Sousa et al., 2022) as solid substrates for SSF processes with *A. niger*, showing the potential of the substrate mixture used in the present work for fungal growth. Moreover, a 9-fold increase in lignocellulosic enzymes was observed from the second to the fourth day of SSF, highlighting xylanase activity that reached 290 U/g after four days of SSF. The fact that OC, which corresponds to 50% of the substrate mixture, have higher content of hemicellulose in comparison to cellulose (Leite et al., 2016), could induce the production of xylanase. Lipase activity increased by 2-fold from the second to the fourth day of SSF, however, this enzyme had the lowest activity detected in these experiments. While the residual oil present in OC could induce lipase production by the filamentous fungi, SSF with *Aspergillus* species for lipase production is often carried for a minimum of seven days (Oliveira et al., 2017, 2016), which explains the low lipase production observed. Moreover, the low protein content in OC may have a negative effect on protease activity since the production of this enzyme by *A. niger* is induced in protein-rich substrates (de Castro et al., 2015).

Table 4.1: Enzymatic activities and free reducing sugars concentration obtained in SSF with *A. niger* CECT 2915 in 50 % (w/w) of OC and SC as solid substrate.

Parameters	**Sample collection (days)**	
	2	**4**
Cellulase (U/g)	7 ± 2^{a}	64 ± 5^{b}
Xylanase (U/g)	32 ± 4^{a}	290 ± 74^{b}
Lipase (U/g)	1.95 ± 0.02^{a}	4 ± 1^{b}
Protease (U/g)	13 ± 3^{a}	10 ± 2^{a}
Reducing sugars (mg/g)	39 ± 3^{a}	18 ± 5^{b}

Values represent the mean and SD from two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).

Regardless of the high activities of the enzymes cellulase and xylanase, the concentration of reducing sugars, which was around 58 mg/g in the substrate mixture prior to *A. niger* inoculation, decline throughout SSF. This result revealed that sugar consumption rate by *A. niger* was higher than the sugar release by the action of the lignocellulosic enzymes, thus an increase in sugars concentration was not detected. Similarly, before *Y. lipolytica* inoculation in pre-fermented okara with the fungi *Rhizopus oligosporus* for 24 h, a decrease in sugars concentration was also observed (Vong et al., 2018a). In contrast, Liu et al., (2018) reported an increase in reducing sugars after 72 h of SSF with *Mucor flavus* using a mixture of okara and buckwheat husk prior to substrate sterilization and *Y. lipolytica* inoculation. Likewise, the release and consumption of reducing sugars by filamentous fungi in SSF is likely dependent of fungal species and the by-products selected as solid substrates. In spite of the reducing sugars consumption by *A. niger*, the fungal growth in the substrate mixture could release other biomolecules, such as proteins for instance (Leite et al., 2016), that could improve yeast growth.

4.3.2 Simultaneous or sequential SSF with *Y. lipolytica* and *A. niger*

Co-culture or sequential SSF with *Y. lipolytica* and filamentous fungi species have been used for substrate biotransformation and biocompound production (Liu et al., 2018b; Vong et al., 2018a). In the present work, two strategies were employed to evaluate the effectiveness of co-culture for lipase production by *Y. lipolytica*: a sequential SSF, where *A. niger* CECT 2915 was first inoculated in the substrate mixture and, after two days of SSF, yeast inoculation was performed, and a simultaneous co-culture with the inoculation of the two microorganisms at the same time.

In the sequential SSF, (8 ± 2) U/g of lipase activity was attained in the second day after yeast inoculation (Table 4.2) and, despite the 2-fold increase in this enzyme activity in comparison to *A. niger* monoculture (Table 4.1), the production in these conditions was very low. Inoculation of the filamentous fungus two days before *Y. lipolytica* decreased free reducing sugars, as shown above, in spite of the carbohydrases production that contribute to assimilable sugars release. However, sugars concentration in the medium is the balance of the sugars released and sugars consumed by microorganisms. The competition for substrate and other nutrients between the yeast cells and the fungus may limit yeast growth and lipase production, since the most active phase of fungal growth seems to be from the second to fourth of fermentation (Table 4.1). For this reason, a simultaneous co-culture was performed and, in these conditions, lipase activity reached (49 ± 7) U/g in the second day of SSF, corresponding to a 6-fold increase compared to the sequential strategy (Table 4.2). It appears that lipase secretion observed in

these experiments is attributed to *Y. lipolytica* since lipase detected after SSF with a monoculture of *A. niger* CECT 2915 for two days was 96% lower (Table 4.1). Moreover, protease activity in the simultaneous co-culture reached (12 ± 2) U/g after two days of SSF and, in the sequential SSF, an increase of 33% in the activity of this enzyme was registered, which could have also contributed to the lower lipase activity detected in these conditions since lipase can be degraded by proteolysis. In the present study, despite the 6-fold improvement on lipase activity comparing the sequential and simultaneous SSF, a standard assay with a monoculture of *Y. lipolytica* W29 was performed for two days and higher lipase activity was achieved, reaching (99 ± 1) U/g, which is similar to the results previously obtained with this substrate mixture (Figure 3.3). These results showed that the mixed cultures of *A. niger* CECT 2915 and *Y. lipolytica* W29 in the co-culture strategies tested in this work did not favor lipase production, thus, application of other pretreatments in the substrate mixture before SSF should be considered.

Table 4.2: Enzymatic activities of lipase and protease obtained in SSF with co-cultures of *A. niger* CECT 2915 and *Y. lipolytica* W29.

Parameters	**SSF**	
	Sequential	**Simultaneous**
Lipase (U/g)	8 ± 2^{a}	49 ± 7^{b}
Protease (U/g)	16 ± 1^{a}	12 ± 2^{b}

Values represent the mean and SD from two independent experiments. Values with different letters within the same row are statistically different ($p < 0.05$)

4.3.3 Enzymatic hydrolysis pretreatment

Since mixed cultures of *Y. lipolytica* W29 and *A. niger* CECT 2915 did not improve lipase production by the oleaginous yeast, a different strategy was used to degrade the cellulose and hemicellulose fractions of the substrate mixture and increase sugar availability prior to *Y. lipolytica* inoculation. For this purpose, enzymatic hydrolysis of the optimum substrate mixture for lipase production was performed using a crude enzymatic extract produced by *A. niger* CECT 2915 and a commercial cellulase was used as a control. The influence of cellulase concentration and incubation time was tested and the time course of reducing sugars release during enzymatic hydrolysis are represented in Figure 4.2.

Overall, an increase in reducing sugars concentration was obtained after only 6 h of hydrolysis in all the conditions tested (Figure 4.2). However, higher sugars release was observed when the enzymatic crude extract from *A. niger* was used in comparison to the commercial cellulase. This commercial enzyme only hydrolyses endo-1,4-β-D-glycosidic linkages in cellulose and cellooligosaccharides. In contrast, the crude enzymatic extract used in this work have different types of cellulases and other hydrolytic enzymes, resulting in increased reducing sugars release. Using the same substrate mixture and filamentous fungus, Sousa et al. (2023) reported that, besides endoglucanases (carboxymethyl cellulases) herein measured, xylanase and β-glucosidase were also found in the aqueous extracts of the fermented mixture.

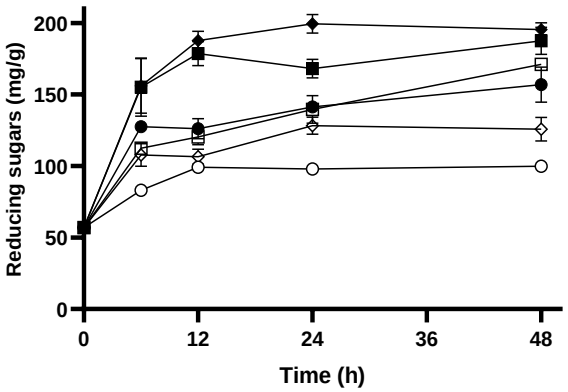

Figure 4.2: Time course of reducing sugars released after enzymatic hydrolysis with a crude enzymatic extract produced by *A. niger* CECT 2915 (filled symbols) and a commercial cellulase (empty symbols) with a cellulase concentration of units per dry mass of substrate adjusted to 50 U/g (●,○), 100 U/g (♦,◊) and 150 (■,□) U/g. The error bars represent the SD of two independent experiments.

Regarding the results using *A. niger* crude extract, sugars release was mainly observed in the first 12 h of the hydrolysis process. Indeed, a 46% increase in reducing sugars concentration was observed when the enzymatic extract amount was adjusted to 100 U/g and 150 U/g of cellulase in comparison to the lowest concentration, corresponding to a 3-fold increase of reducing sugars in the substrate mixture after 12 h of treatment. Using a crude enzymatic extract produced by *A. niger*, Martínez-Avila et al. (2021) performed enzymatic hydrolysis with high solid loading of three agro-industrial by-products, including OC. While sugars release was observed in brewer's spent grain and grape pomace, enzymatic hydrolysis of OC did not improve the reducing sugars content of this by-product. Moreover, the authors reported that the high lignin content in OC could play a critical role in substrate hydrolysis. In the present study, the substrate submitted to enzymatic hydrolysis is a mixture of two oil cakes with different chemical

composition. In particular, while the cellulose content in these materials is very similar, lignin percentage in SC is 81% lower than in OC (Leite et al., 2016; Sousa et al., 2022), which could improve the release of reducing sugars and increase their availability in the substrate mixture. Since increasing cellulase concentration to 150 U/g or extending enzymatic hydrolysis above 12 h did not increase sugars release, the crude enzymatic extract with a cellulase concentration of 100 U/g and 12 h of incubation time were selected for further experiments. After 12 h of incubation with the crude enzymatic extract, fiber content decreased 20% in the substrate mixture (Table 4.3), showing that the enzymatic extract obtained by SSF with *A. niger* effectively degraded the lignocellulosic matrix of the mixture of OC and SC. This outcome is in accordance with reports showing a reduction in fiber content after SSF with *Aspergillus* species (Salgado et al., 2015; Sousa et al., 2022). Additionally, enzymatic hydrolysis resulted in a 42% increase in glucose in the aqueous extracts, and a 3-fold increase in xylose concentration in comparison to the substrate mixture without pretreatment (Table 4.3). The differences in the release of these sugars could be related to the production of high levels of xylanase by *A. niger* in the ternary mixture of oil cakes used for the enzymatic crude extract production (Sousa et al., 2023). Hemicellulose degradation by xylanases could also justify the significant decrease in NDF (Table 4.3), while the decrease in ADF from the untreated to the hydrolyzed substrate mixture, is statistically significant with a confidence level of 94%.

Table 4.3: Characterization of the substrate mixture pretreated with a crude enzymatic extract for 12 h with a cellulase concentration of 100 U/g.

Parameters	**Time (h)**	
	0	**12**
NDF (%, w/w)	52 ± 1[a]	41 ± 3[b]
ADF (%, w/w)	35 ± 2[a]	28 ± 1[a]
Glucose (mg/g)*	36 ± 8[a]	51 ± 1[b]
Xylose (mg/g)*	9 ± 2[a]	26 ± 1[b]

* Sugar quantified in the aqueous extract after extraction of the pre-hydrolyzed substrate mixture.

NDF: neutral detergent fiber; ADF: acid detergent fiber.

Values represent the mean and SD from two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).

In the present study, enzymatic hydrolysis was successfully performed with a high solid loading of 25% (w/w, wet basis), resulting in a 3-fold increase in free reducing sugars in the substrate mixture. Water is essential in hydrolysis reactions and increasing the solid loading in enzymatic hydrolysis of lignocellulosic biomass may have a negative impact on the conversion of the lignocellulosic matrix into fermentable sugars (Modenbach and Nokes, 2013). However, the implementation of enzymatic hydrolysis processes with high solid loadings have additional advantages, such as low water usage and reduction in the production costs (Modenbach and Nokes, 2013). The fact that the moisture content used in enzymatic hydrolysis was similar to that used in SSF with *Y. lipolytica* W29 allowed the substrate utilization with minimal processing steps.

4.3.4 SSF of enzymatically pretreated substrate

After selection of the optimum conditions for the enzymatic hydrolysis, pre-hydrolyzed mixture of OC and SC was fermented with *Y. lipolytica* W29 for two days, the incubation time for lipase production previously optimized in Chapter 3, and the lipase activity registered in these conditions was very low (Table 4.4). While distilled water was used to adjust the moisture content in the substrate mixture of OC and SC in SSF for lipase production in the experiments reported so far, in these experiments, citrate buffer with a pH of 4.8 was used to guarantee suitable conditions for enzymatic hydrolysis before SSF. Thus, to examine if the low lipase activity observed after enzymatic hydrolysis was related to the chances in the substrate mixture pH, SSF was performed in the mixture of OC and SC without enzymatic hydrolysis using water and citrate buffer (pH 4.8) to adjust the moisture content.

Table 4.4: Lipase activity, cellular density and pH values obtained after SSF for two days with a mixture of OC and SC with and without enzymatic hydrolysis prior to *Y. lipolytica* W29 inoculation.

Parameters	**Pretreated substrate**	**Unpretreated substrate**	
		Water	**Citrate buffer pH 4.8**
Lipase activity (U/g)	5 ± 1^{a}	78 ± 2^{b}	60 ± 6^{c}
Cellular density (mg/g)	43 ± 3^{a}	18 ± 2^{b}	15 ± 2^{b}
pH	5.39 ± 0.04^{a}	6.8 ± 0.1^{b}	5.8 ± 0.1^{c}

Values represent the mean and SD from two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).

As expected, significant lower pH values were observed when citrate buffer was used to moisten the substrate mixture compared to SSF that utilized water for this purpose (Table 4.4). It appears that this parameter had some influence on lipase secretion since a 29% reduction in lipase activity was observed when citrate buffer was used instead of water in SSF with the untreated substrate mixture. However, an 11-fold decrease in lipase activity was attained when enzymatic hydrolysis was performed before SSF compared with the untreated mixture (moistened with citrate buffer), revealing that low pH in these fermentations may not be the only reason for low lipase detection. Another aspect considered in these experiments was the effect of enzymatic hydrolysis on the cell concentration obtained after SSF. Although a similar cellular growth was observed in SSF without enzymatic pretreatment, an almost 3-fold increase in yeast cell concentration was observed when the substrate mixture was pretreated with the fungal enzymatic extract. This outcome could be related to the increased reducing sugars available in the substrate mixture, which improved cellular growth in detriment of biocompound production. To further understand the effect of enzymatic hydrolysis on lipase production, kinetics of SSF was performed up to 5 days (Figure 4.3).

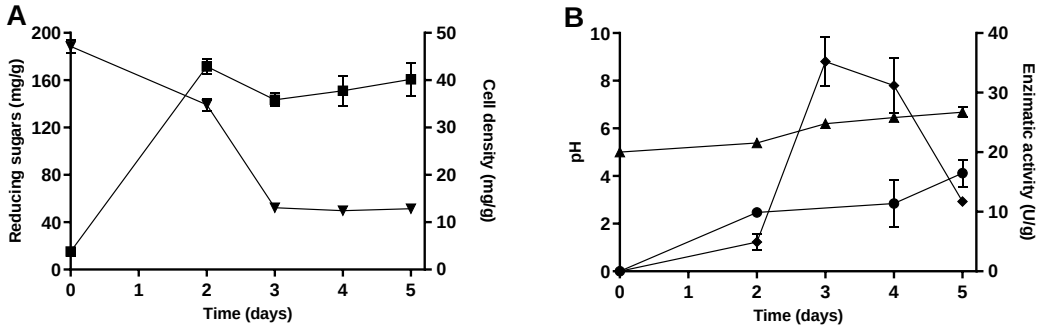

Figure 4.3: Time course of cellular density (■), reducing sugars concentration (▼) (A), activities of lipase (♦) and protease (●) and pH (▲) (B) obtained during SSF with *Y. lipolytica* W29 with a 50% (w/w) mixture of OC and SC pretreated with an enzymatic extract produced by *A. niger*. The error bars represent the SD of two independent experiments.

An 11-fold increase in cellular concentration was observed in the second day of fermentation and cellular growth stabilized until the end of the experiments (Figure 4.3A). Comparing these results with the kinetics for lipase production without enzymatic hydrolysis of the substrate mixture (Figure 3.3), enzymatic hydrolysis resulted in a 42% increase in yeast biomass in the fourth day of SSF, which corresponded to

the end of SSF in the experiments with the untreated substrate. Likewise, the 3-fold increase in reducing sugars concentration in the substrate mixture after enzymatic hydrolysis improved *Y. lipolytica* growth. The higher sugars availability in the substrate mixture allowed *Y. lipolytica* to metabolize around 137 mg/g of reducing sugars, corresponding to a 3-fold increase in sugar consumption in comparison to SSF without substrate hydrolysis (Figure 3.3A). After the third day of SSF, a residual value of reducing sugars was maintained until the end of the experiments. Even though a residual value of sugars was also previously observed (Figure 3.3A), the content of reducing sugars detected at the end of these experiments was 5 times higher, possibly corresponding to monomers that were release during enzymatic hydrolysis and that *Y. lipolytica* is unable to efficiently assimilate. Concerning lipase activity, a 7-fold increase was observed between the second and third days of SSF (Figure 4.3B). After reaching its maximum on the third day of cultivation, lipase activity decreased and, by the end of SSF, 34% of this enzyme activity was still detected. In these conditions, a decrease in lipase productivity (U/g/day) was observed since the maximum activity was attained later in the SSF process. The delayed peak could be explained by the higher concentration of reducing sugars in the SSF medium since lipase secretion into extracellular medium occurs when the concentration of carbon sources are low (Lopes et al., 2016). Moreover, a reduction in maximum lipase activity was observed with the substrate hydrolysis (Figure 3.3B, Figure 4.3B, Table 4.4). Once again, this result could be related to the higher concentration of sugars in the substrate mixture. Najjar et al., (2011) observed that in medium containing both glucose and olive oil, lipase production peak was delayed and lower values were obtained compared to medium only supplemented with olive oil. While *LIP2,* the gene coding for the most produced extracellular lipase in *Y. lipolytica*, is induced in the presence of oily substrates, it appears that the presence of glucose have a repressive effect in this gene expression (Fickers et al., 2011). In fact, Fickers et al. (2005) observed that hexokinase, responsible for the phosphorylation of hexoses in the early stage of the glycolytic pathway, is involved in the repression of *LIP2* in *Y. lipolytica.* Even though glucose was present in the substrate mixture before enzymatic hydrolysis, the 42% increase in glucose concentration could be sufficient to alter *Y. lipolytica* metabolism. Moreover, the increase of other reducing sugars' concentration besides glucose could also play a role in the decreased lipase production observed in this work. As presented in Chapter 3, pH values increased until the end of cultivation with the untreated substrate mixture reaching values above 8 (Figure 3.3B), however, pH in the present study was kept bellow 7 until the end of SSF. Despite these differences, the enzymatic profiles obtained with the untreated mixture of OC and SC (Figure 3.3B) and after enzymatic hydrolysis (Figure 4.3B) were very similar, with the lipase activity peak preceding the increase in protease activity. Furthermore, an increase in medium pH is often

related to protease release, leading to lipase degradation by proteolysis both in solid state and submerged fermentations (Braga et al., 2012; Souza et al., 2017). Despite the lower pH observed in this work, protease activity was also detected in the aqueous extract obtained after SSF (Figure 4.3B). Nonetheless, the production was lower in comparison to the results obtained with the untreated substrate mixture (Figure 3.3B). *Yarrowia lipolytica* secretes two enzymes with proteolytic activity and their secretion is influenced by the medium pH. While the most secreted protease is induced by alkaline pH, an acid protease is also secreted by the oleaginous yeast (Ogrydziak, 2013). Thus, the lower pH after enzymatic hydrolysis still induced protease production by *Y. lipolytica* and the activity of this enzyme increased until the end of SSF possibly resulting in lipase degradation.

Regardless of the lower lipase activity detected in the pretreated mixture of 50% (w/w, dry basis) of OC and SC, the results obtained in this study showed the great potential of employing crude extracts produced by filamentous fungi in enzymatic hydrolysis processes without any enzyme purification steps. Furthermore, these conditions favored yeast biomass production, which could result in oil cakes biotransformation and improvement of the nutritional value of these materials enriched with microbial protein. Moreover, the fact that the hydrolysis was successfully performed in a high-solid loading system allowed the obtention of a solid substrate mixture with increased free and easily assimilable sugars, through an economic and eco-friendly process.

4.3.5 Solid state and semi solid fermentations after physical pretreatments

Physical pretreatments are often applied prior to enzymatic hydrolysis, resulting in higher yields of fermentable sugars that can be subsequently used in biotechnological processes for value-added compounds production (Cantero et al., 2019). However, these pretreatments can also be beneficial before SSF since the changes in the solid substrate can increase the access of microorganisms to soluble compounds and microbial enzymes to their substrates. Thus, in the present study, the substrate mixture of OC and SC with a solid loading of 25% (w/w, dry basis) was placed in an ultrasonic bath for 15 min or was submitted to microwave irradiation for 2 min before SSF for 2 days with *Y. lipolytica* W29 (Table 4.5).

Microwave and ultrasound pretreatment resulted in a 10% and 16% increase in reducing sugars content in the substrate mixture, respectively, in comparison to the untreated mixture (Table 4.5), although the values are not statistically different. These values are significant lower in comparison to the sugar release observed after enzymatic hydrolysis (Figure 4.2) since the severity of the pretreatments was not enough to hydrolyze the polysaccharides from lignocellulosic matrix.

Table 4.5: Effect of physical pretreatments on the release of reducing sugars before SSF, cellular growth and enzyme production by *Y. lipolytica* W29 after 2 days of SSF.

Parameters	Physical pre-treatment		Untreated substrate
	Ultrasound	Microwave	
Reducing sugars released (mg/g)	67 ± 6[a]	64 ± 1[a]	58 ± 1[a]
Cellular concentration (mg/g)	18 ± 2[a]	26 ± 6[b]	18 ± 1[a]
Maximum lipase production (U/g)	56 ± 9[a,b]	63 ± 4[b]	54 ± 2[a]
Protease production (U/g)	9 ± 3[a]	3.24 ± 0.01[a]	6 ± 2[a]

Values represent the mean and SD from two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).

Ultrasound pretreatment had no effect on cellular growth, however, when the substrate mixture was irradiated with microwaves, a 44% increase in this parameter was detected (Table 4.5). Moreover, the pretreatments used in this work improved lipase production, highlighting the results obtained with microwave pretreatment, which resulted in a 1.2-fold enzyme activity increase in comparison to SSF with the untreated substrate mixture. Zhao et al. (2010) reported that a 3 min microwave pretreatment of rice hulls and wheat bran before SSF did not significantly alter cellulase production by *Trichoderma* sp. Additionally, the authors reported that improved enzyme production was only achieved when microwave was combined with an alkaline pretreatment. In the present work, microwave pretreatment was enough to increase lipase production by *Y. lipolytica* W29, resulting in less processing of the solid materials after pretreatment and before SSF. Regarding protease activity, the pretreatments had no effect on this enzyme activity since the differences observed are not statistically significant.

In all the experiments involving biological and physical pretreatments in this work, a standard SSF assay for 2 days without any substrate pretreatment was performed and used as a control condition. Taking into account the results previously obtained in Chapter 3 and the three standard assays performed in the present Chapter, lipase activity varied between 102 U/g (Figure 3.3B) and 52 U/g (Table 4.5), thus, the activity of this enzyme in the untreated substrate mixture of OC and SC was, on average, (82 ± 24) U/g. The high standard deviation observed in these experiments could be related to the fact that the oil cakes are very heterogenous and the percentage of lipids, acting as lipase inducers, in the substrate mixture could differ throughout these experiments.

Besides pretreatment with an ultrasonic bath, the substrate mixture at a solid loading of 10% (w/w) in water suspension was also pretreated with an ultrasonic probe. This semi-solid mixture was

composed of 10.8 g/L of reducing sugars and the ultrasonic irradiation led to a slight increase in sugars content (12.2 g/L) after 6 min of pretreatment and extending pretreatment time to 10 and 15 min did not improve reducing sugars release. Although the sonication did not result in significant changes in the reducing sugars concentration in the mixture of OC and SC, it is possible that this pretreatment improved the accessibility of *Y. lipolytica* W29 to other biomolecules present in the substrate. Thus, ultrasound pretreatment for 6 min was applied to the substrate mixture before yeast inoculation. As previously stated, to perform these experiments, the amount of water added to the substrate was higher than in the experiments of SSF, resulting in a semi-solid mixture of OC and SC. Moreover, to minimize the complexity of the bioprocess, which would require the drying of the pretreated substrate followed by moisture adjustment before SSF, the semi-solid mixture was directly inoculated with *Y. lipolytica* W29 after a sterilization step and fermentations were carried for 30 h (Figure 4.4). A semi-solid fermentation with an untreated semi-solid mixture was performed as a control.

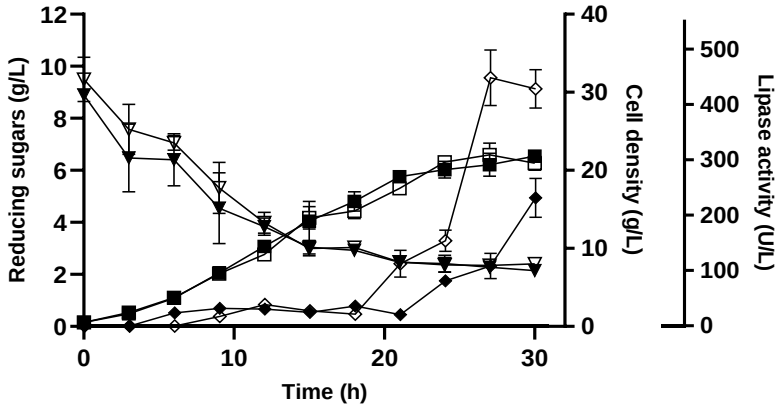

Figure 4.4: Time course of cell growth (■,□), reducing sugars concentration (▼,▽) and lipase activity (♦,◊) after semi-solid fermentation with a 50 % (w/w, dry basis) mixture of OC and SC pretreated with ultrasonic irradiation for 6 min (empty symbols) and the control assays without substrate pretreatment (filled symbols) for 30 h. The error bars represent the SD of two independent experiments.

The majority of reducing sugars consumption occurred in the first 15 h of cultivation in both untreated and pretreated mixtures (Figure 4.4). Similar to what occurred in SSF (Figure 4.3B), a residual value of reducing sugars was detected until the end of the fermentation process. Moreover, ultrasound pretreatment did not result in changes in yeast cell growth compared to the untreated semi-solid mixture. In particular, after 30 h of cultivation, yeast cells had already entered stationary phase and a final cell

biomass of around 20 g/L was obtained in both conditions tested. In SSF after ultrasonic pretreatment (Table 4.5), the 18 mg/g of yeast biomass obtained after two days of SSF corresponds to 2.3 g/L, thus, an almost 10-fold increase in cell concentration was achieved in semi-solid fermentation. In these experiments, oxygen transfer was favored by medium agitation, which could explain the high yeast biomass production in semi-solid fermentation in comparison to SSF. Moreover, the high amount of water in these conditions and the agitation of the slurry medium could increase solubilization of the substrate mixture also increasing bioavailability of compounds that could be metabolized by the yeast. Besides reducing sugars and lipids, *Y. lipolytica* may also metabolize phenolic compounds present in the oil cakes used in these experiments (Table 3.1). In fact, this yeast have been used to reduce the content of phenolic compounds in OMWW (Gonçalves et al., 2009; Sarris et al., 2011). Additionally, other compounds such as alcohols and organic acids could be present in this substrate mixture, contributing to *Y. lipolytica* growth. Lipase activity quantification was performed and, regardless of the pretreatment used in the semi-solid mixture, lipase secretion occurred mostly at the end of these experiments (Figure 4.4). These results are in agreement with Pereira-Meirelles et al. (2000), which showed that lipase secretion to the culture medium by *Y. lipolytica* begins when cells enter the stationary phase and carbon sources become limited. In the control condition, lipase activity increased until the end of semi-solid fermentation, reaching its maximum value of 233 U/L. Conversely, ultrasound pretreatment improved lipase activity, which peaked after 27 h of fermentation and was 2-fold higher than the maximum lipase activity detected in the control assay. Thus, it is possible that the application of ultrasounds in the mixture of OC and SC could increase the bioavailability of lipids in the substrate mixture and the fact that, in these experiments, the semi-solid mixture was constantly agitated, improved the access of *Y. lipolytica* to these lipase inducers. Protease activity was quantified at the end of these experiments and 1576 U/L were obtained with the untreated semi-solid mixture. Furthermore, protease activity in the experiments with pretreated semi-solid mixture was 21% lower, which could also contribute to the higher lipase activity detected in these conditions. After semi-solid fermentation, the content of the flasks was dried without yeast biomass separation and the remaining solid was characterized to understand the influence of *Y. lipolytica* growth in substrate composition (Table 4.6).

Table 4.6: Effect of semi-solid fermentation on crude protein, total lipids and lipids composition on LCFAs.

Parameter		Unfermented	Semi-solid fermentation	
			Pretreated by US	Untreated
Crude protein (%, w/w)		22 ± 1[a]	20 ± 1[a]	20 ± 1[a]
Lipids (%, w/w)		6.1 ± 0.4[a]	3 ± 1[b]	3.0 ± 0.3[b]
LCFAs (%)	C16:0	14 ± 1[a]	12.2 ± 0.4[b]	12.1 ± 0.4[b]
	C16:1	0.6 ± 0.1[a]	2.0 ± 0.2[b]	2.1 ± 0.3[b]
	C17:1	0.2 ± 0.1[a]	ND	0.2 ± 0.1[a]
	C18:0	2 ± 1[a]	1.9 ± 0.4[a]	1.9 ± 0.4[a]
	C18:1	62 ± 1[a]	59.3 ± 0.4[a]	59.7 ± 0.8[a]
	C18:2	21 ± 1[a]	25 ± 1[b]	24 ± 1[b]

Values represent the mean and SD from two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).
LCFA: long chain fatty acids; ND: not detected

While yeast growth had no impact on crude protein content, a 50% reduction on lipid content was observed after 30 h of incubation compared to the mixture without inoculation (0 h). Lipase production by *Y. lipolytica* and the ability of these enzymes to hydrolyze triacylglycerol (Lopes et al., 2022) can explain the decrease in lipid content in the fermented solid. As previously stated, growth of *Y. lipolytica* W29 resulted in a 29% reduction in SSF with this substrate mixture (Table 3.3), showing that in semi-solid fermentation yeast cells were able to reduce the lipidic content of the substrate mixture more effectively in a shorter period of time. Moreover, SSF with *Y. lipolytica* NBRC-10073 resulted in a lipid content reduction in fish by-products, demonstrating the potential of this microorganism for by-products biotransformation (Yano et al., 2008). Moreover, the profile of long chain fatty acids (LCFAs) were also analyzed after semi-solid fermentation (Table 4.6). The substrate mixture composed of OC and SC used in this work is characterized by a high oleic acid (C18:1) content, a fatty acid present in high percentages in by-products obtained after olive oil extraction (Molina-Alcaide and Yáñez-Ruiz, 2008). Moreover, this value is followed by linoleic acid (C18:2), showing that more than 80% of the fatty acids in this substrate mixture are unsaturated fatty acids. Semi-solid fermentation resulted in a 3.3 and 3.5-fold increase in palmitoleic acid (C16:1) in the substrate mixture treated with ultrasounds and in the untreated one, respectively. Moreover, yeast cultivation for 30 h also resulted in around 16.5% increase in linoleic acid. Although the contents of heptadecanoic acid (C17:1), stearic acid (C18:0) and oleic acid were unaffected

by semi-solid fermentation, this bioprocess resulted in a 14% reduction in palmitic acid (C16:0) percentage.

Solid state fermentation can be used to improve the nutritional properties of agro-industrial by-products before their incorporation into animal feed formulations (Sousa et al., 2022). Moreover, oilseeds are often used as fat supplementation in some feedstocks to increase the intake of some unsaturated fatty acids, such as oleic and linoleic acids (Enjalbert et al., 2017). In the present study, a slight increase in the percentage of unsaturated fatty acids was observed regardless of the utilization of ultrasonic pretreatment. Furthermore, since the content of saturated fatty acids suffered a 10% reduction, the fermented substrate presented a higher ratio of unsaturated to saturated fatty acids, resulting in a nutritional enhancement of the mixture of oil cakes and showing its potential application in animal feed formulations.

4.4 Conclusions

In the present Chapter, several biological and physical pretreatments were used to increase sugars availability and substrate accessibility and their effect on yeast growth and lipase production was evaluated. The co-culture strategies employed in this work did not improve lipase production by *Y. lipolytica* W29. Enzymatic hydrolysis at high solid loading of the 50 % (w/w, dry basis) mixture of OC and SC resulted in high reducing sugars release, which were effectively used by *Y. lipolytica* W29 in SSF, improving yeast growth in comparison to the standard experiment with the untreated substrate mixture. Moreover, physical pretreatments before SSF had a positive effect on lipase production, with a highlight to microwave irradiation that not only led to an increase in lipase activity but also resulted in improved cellular growth. Semi-solid fermentation was successfully performed with this substrate mixture and ultrasonic pretreatment led to a 2-fold increase in lipase production. Yeast growth in semi-solid fermentation increased the percentage of unsaturated fatty acids and, on the other hand, a reduction of saturated fatty acids was observed, thus, the lipidic profile of the substrate mixture was improved.

4.5 REFERENCES

Braga, A., Gomes, N., Belo, I., 2012. Lipase induction in *Yarrowia lipolytica* for castor oil hydrolysis and its effect on γ-decalactone production. J. Am. Oil Chem. Soc. 89, 1041–1047. https://doi.org/10.1007/s11746-011-1987-5

Cantero, D., Jara, R., Navarrete, A., Pelaz, L., Queiroz, J., Rodríguez-Rojo, S., Cocero, M.J., 2019. Pretreatment processes of biomass for biorefineries: Current status and prospects. Annu. Rev. Chem. Biomol. Eng. 10, 289–310. https://doi.org/10.1146/annurev-chembioeng-060718-030354

Costa, A.R., Salgado, J.M., Lopes, M., Belo, I., 2022. Valorization of by-products from vegetable oil industries: Enzymes production by *Yarrowia lipolytica* through solid state fermentation. Front. Sustain. Food Syst. 6. https://doi.org/10.3389/fsufs.2022.1006467

de Castro, R.J.S., Ohara, A., Nishide, T.G., Albernaz, J.R.M., Soares, M.H., Sato, H.H., 2015. A new approach for proteases production by Aspergillus niger based on the kinetic and thermodynamic parameters of the enzymes obtained. Biocatal. Agric. Biotechnol. 4, 199–207. https://doi.org/10.1016/j.bcab.2014.12.001

Enjalbert, F., Combes, S., Zened, A., Meynadier, A., 2017. Rumen microbiota and dietary fat: a mutual shaping. J. Appl. Microbiol. 123, 782–797. https://doi.org/10.1111/jam.13501

Farias, M.A., Valoni, E.A., Castro, A.M., Coelho, M.A.Z., 2014. Lipase production by *Yarrowia lipolytica* in solid state fermentation using different agro industrial residues. Chem. Eng. Trans. 38, 301–306. https://doi.org/10.3303/CET1438051

Ferreira, M., Fernandes, H., Peres, H., Oliva-Teles, A., Belo, I., Salgado, J.M., 2020. Bio-enrichment of oilseed cakes by *Mortierella alpina* under solid-state fermentation. LWT - Food Sci. Technol. 134. https://doi.org/10.1016/j.lwt.2020.109981

Fickers, P., Marty, A., Nicaud, J.M., 2011. The lipases from *Yarrowia lipolytica*: Genetics, production, regulation, biochemical characterization and biotechnological applications. Biotechnol. Adv. 29, 632–644. https://doi.org/10.1016/j.biotechadv.2011.04.005

Fickers, P., Nicaud, J.M., Destain, J., Thonart, P., 2005. Involvement of hexokinase Hxk1 in glucose catabolite repression of LIP2 encoding extracellular lipase in the yeast *Yarrowia lipolytica*. Curr. Microbiol. 50, 133–137. https://doi.org/10.1007/s00284-004-4401-9

Filipe, D., Fernandes, H., Castro, C., Peres, H., Oliva-Teles, A., Belo, I., Salgado, J.M., 2020. Improved lignocellulolytic enzyme production and antioxidant extraction using solid-state fermentation of olive pomace mixed with winery waste. Biofuels, Bioprod. Biorefining 14, 78–91. https://doi.org/10.1002/bbb.2073

Gonçalves, C., Lopes, M., Ferreira, J.P., Belo, I., 2009. Biological treatment of olive mill wastewater by non-conventional yeasts. Bioresour. Technol. 100, 3759–3763. https://doi.org/10.1016/j.biortech.2009.01.004

Imandi, S.B., Garapati, H.R., 2007. Lipase Production by *Yarrowia lipolytica* NCIM 3589 in Solid State Fermentation Using Mixed Substrate. Res. J. Microbiol.

https://doi.org/10.3923/jm.2007.469.474

Imandi, S.B., Karanam, S.K., Garapati, H.R., 2010. Optimization of media constituents for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from palm Kernal cake (Elaeis guineensis). Adv. Biosci. Biotechnol. 1, 115–121. https://doi.org/10.4236/abb.2010.12016

Leite, P., Salgado, J.M., Venâncio, A., Domínguez, J.M., Belo, I., 2016. Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. Bioresour. Technol. 214, 737–746. https://doi.org/10.1016/j.biortech.2016.05.028

Leite, P., Sousa, D., Fernandes, H., Ferreira, M., Costa, A.R., Filipe, D., Gonçalves, M., Peres, H., Belo, I., Salgado, J.M., 2021. Recent advances in production of lignocellulolytic enzymes by solid-state fermentation of agro-industrial wastes. Curr. Opin. Green Sustain. Chem. 27, 100407. https://doi.org/10.1016/j.cogsc.2020.100407

Liu, X., Yu, X., Zhang, T., Wang, Z., Xu, Jiaxing, Xia, J., He, A., Yan, Y., Xu, Jiming, 2018. Novel two-stage solid-state fermentation for erythritol production on okara–buckwheat husk medium. Bioresour. Technol. 266, 439–446. https://doi.org/10.1016/j.biortech.2018.07.009

Lopes, M., Miranda, S.M., Alves, J.M., Pereira, A.S., Belo, I., 2018. Waste Cooking Oils as Feedstock for Lipase and Lipid-Rich Biomass Production. Eur. J. Lipid Sci. Technol. 1–9. https://doi.org/10.1002/ejlt.201800188

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. Crit. Rev. Biotechnol. 42, 163–183. https://doi.org/10.1080/07388551.2021.1931016

Lopes, V.R.O., Farias, M.A., Belo, I.M.P., Coelho, M.A.Z., 2016. Nitrogen sources on TPOMW valorization through solid state fermentation performed by *Yarrowia lipolytica*. Brazilian J. Chem. Eng. 33, 261–270. https://doi.org/10.1590/0104-6632.20160332s20150146

Martínez-Avila, O., Llimós, J., Ponsá, S., 2021. Integrated solid-state enzymatic hydrolysis and solid-state fermentation for producing sustainable polyhydroxyalkanoates from low-cost agro-industrial residues. Food Bioprod. Process. 126, 334–344. https://doi.org/10.1016/j.fbp.2021.01.015

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426–428.

Modenbach, A.A., Nokes, S.E., 2013. Enzymatic hydrolysis of biomass at high-solids loadings - A review. Biomass and Bioenergy 56, 526–544. https://doi.org/10.1016/j.biombioe.2013.05.031

Moftah, O.A.S., Grbavčić, S., Žuža, M., Luković, N., Bezbradica, D., Knežević-Jugović, Z., 2012. Adding value to the oil cake as a waste from oil processing industry: Production of lipase and protease by *Candida utilis* in solid state fermentation. Appl. Biochem. Biotechnol. 166, 348–364. https://doi.org/10.1007/s12010-011-9429-2

Moftah, O.A.S., Grbavcic, S.Z., Moftah, W.A.S., Lukovic, N.D., Prodanovic, O.L., Jakovetic, S.M., Kneževic-Jugovic, Z.D., 2013. Lipase production by *Yarrowia lipolytica* using olive oil processing wastes as substrates. J. Serbian Chem. Soc. 78, 781–794. https://doi.org/10.2298/JSC120905005M

Molina-Alcaide, E., Yáñez-Ruiz, D.R., 2008. Potential use of olive by-products in ruminant feeding: A review. Anim. Feed Sci. Technol. 147, 247–264. https://doi.org/10.1016/j.anifeedsci.2007.09.021

Moran-Aguilar, M.G., Costa-Trigo, I., Calderón-Santoyo, M., Domínguez, J.M., Aguilar-Uscanga, M.G., 2021. Production of cellulases and xylanases in solid-state fermentation by different strains of *Aspergillus niger* using sugarcane bagasse and brewery spent grain. Biochem. Eng. J. 172. https://doi.org/10.1016/j.bej.2021.108060

Najjar, A., Robert, S., Guérin, C., Violet-Asther, M., Carrière, F., 2011. Quantitative study of lipase secretion, extracellular lipolysis, and lipid storage in the yeast *Yarrowia lipolytica* grown in the presence of olive oil: Analogies with lipolysis in humans. Appl. Microbiol. Biotechnol. 89, 1947–1962. https://doi.org/10.1007/s00253-010-2993-5

Ogrydziak, D., 2013. Acid and Alkaline Extracellular Proteases of *Yarrowia lipolytica*, in: Barth G. (Eds) Yarrowia Lipolytica. Microbiology Monographs. https://doi.org/10.1007/978-3-642-38583-4

Oliveira, F., Moreira, C., Salgado, J.M., Abrunhosa, L., Venâncio, A., Belo, I., 2016. Olive pomace valorization by *Aspergillus* species: lipase production using solid-state fermentation. J. Sci. Food Agric. 96, 3583–3589. https://doi.org/10.1002/jsfa.7544

Oliveira, F., Salgado, J.M., Abrunhosa, L., Pérez-Rodríguez, N., Domínguez, J.M., Venâncio, A., Belo, I., 2017. Optimization of lipase production by solid-state fermentation of olive pomace: from flask to laboratory-scale packed-bed bioreactor. Bioprocess Biosyst. Eng. 40, 1123–1132. https://doi.org/10.1007/s00449-017-1774-2

Ong, V.Z., Wu, T.Y., 2020. An application of ultrasonication in lignocellulosic biomass valorisation into bio-energy and bio-based products. Renew. Sustain. Energy Rev. 132, 109924. https://doi.org/10.1016/j.rser.2020.109924

Pereira-Meirelles, F. V., Rocha-Leão, M.H.M., Sant'Anna, G.L., 2000. Lipase location in *Yarrowia lipolytica* cells. Biotechnol. Lett. 22, 71–75. https://doi.org/10.1023/A:1005672731818

Sadh, P.K., Duhan, S., Duhan, J.S., 2018. Agro-industrial wastes and their utilization using solid state fermentation: a review. Bioresour. Bioprocess. 5, 1–15. https://doi.org/10.1186/s40643-017-0187-z

Salgado, J.M., Abrunhosa, L., Venâncio, A., Domínguez, J.M., Belo, I., 2015. Enhancing the Bioconversion of Winery and Olive Mill Waste Mixtures into Lignocellulolytic Enzymes and Animal Feed by *Aspergillus uvarum* Using a Packed-Bed Bioreactor. J. Agric. Food Chem. 63, 9306–9314. https://doi.org/10.1021/acs.jafc.5b02131

Sarris, D., Galiotou-Panayotou, M., Koutinas, A.A., Komaitis, M., Papanikolaou, S., 2011. Citric acid, biomass and cellular lipid production by *Yarrowia lipolytica* strains cultivated on olive mill wastewater-based media. J. Chem. Technol. Biotechnol. 86, 1439–1448. https://doi.org/10.1002/jctb.2658

Sousa, D., Salgado, J.M., Cambra-López, M., Dias, A., Belo, I., 2023. Biotechnological valorization of oilseed cakes: Substrate optimization by simplex centroid mixture design and scale-up to tray bioreactor. Biofuels, Bioprod. Biorefining 17, 121–134. https://doi.org/10.1002/bbb.2428

Sousa, D., Salgado, J.M., Cambra-López, M., Dias, A.C.P., Belo, I., 2022. Degradation of lignocellulosic matrix of oilseed cakes by solid-state fermentation: fungi screening for enzymes production and antioxidants release. J. Sci. Food Agric. 102, 1550–1560. https://doi.org/10.1002/jsfa.11490

Souza, C., Farias, M.A., Ribeiro, B.D., Coelho, M.A.Z., 2017. Adding Value to Agro-industrial Co-products from Canola and Soybean Oil Extraction Through Lipase Production Using *Yarrowia lipolytica* in Solid-State Fermentation. Waste and Biomass Valorization 8, 1163–1176.

Swain, M.R., Mishra, J., Thatoi, H., 2013. Bioethanol Production from Sweet Potato (*Ipomoea batatas* L.) Flour using Co-Culture of *Trichoderma* sp. and *Saccharomyces cerevisiae* in Solid-State Fermentation. Brazilian Arch. Biol. Technol. 56, 171–179. https://doi.org/10.1590/S1516-89132013000200002

Try, S., De-Coninck, J., Voilley, A., Chunhieng, T., Waché, Y., 2018. Solid state fermentation for the production of γ-decalactones by *Yarrowia lipolytica*. Process Biochem. 64, 9–15. https://doi.org/10.1016/j.procbio.2017.10.004

Vong, W.C., Hua, X.Y., Liu, S.Q., 2018. Solid-state fermentation with *Rhizopus oligosporus* and *Yarrowia lipolytica* improved nutritional and flavour properties of okara. LWT - Food Sci. Technol. 90, 316–322. https://doi.org/10.1016/j.lwt.2017.12.050

Yano, Y., Oikawa, H., Satomi, M., 2008. Reduction of lipids in fish meal prepared from fish waste by a yeast *Yarrowia lipolytica*. Int. J. Food Microbiol. 121, 302–307. https://doi.org/10.1016/j.ijfoodmicro.2007.11.012

Zhao, X., Zhou, Y., Zheng, G., Liu, D., 2010. Microwave pretreatment of substrates for Cellulase production by solid-state fermentation. Appl. Biochem. Biotechnol. 160, 1557–1571. https://doi.org/10.1007/s12010-009-8640-x

5 OLIVE AND SUNFLOWER CAKES AS SUITABLE SUBSTRATES FOR LIPASE PRODUCTION BY *YARROWIA* SPP.: FROM FLASKS TO BIOREACTOR

SSF is a biotechnological process that allows the utilization of agro-industrial by-products as solid substrates for the growth of microorganisms, such as filamentous fungi and yeasts, with the simultaneous production of added value compounds. Oil cakes, such as OC and SC, are generated during vegetable oil extraction and have received great interest as solid substrates in SSF processes. In the present Chapter, a 50 % (w/w) mixture of OC and SC was used as substrate in SSF for lipase production by several strains of *Y. lipolytica* and *Y. divulgata*. The strain of *Y. lipolytica* W29 was the best lipase producer under small scale SSF and studies were conducted to address important factors in scaling-up the process. Different bioreactors were used and the effect of aeration and agitation on lipase production was studied. Higher lipase activity was obtained in tray bioreactors, reaching 85 U/g (dry mass of substrates) after 48 h of SSF, while the operation in a horizontal drum bioreactor favored yeast growth, resulting in highest yeast biomass production and a delay on lipase maximum activity. The scale-up of SSF for lipase production by *Y. lipolytica* has been successfully proven, thus this is an interesting and eco-friendly bioprocessing strategy for valorization of by-products from vegetable oil industries.

This chapter is based on the following research article:

Costa, A.R., Salgado, J.M., Belo, I., 2023. Olive and sunflower cakes as suitable substrates for lipase production by *Yarrowia* spp.: from flasks to bioreactor. Biocatal. Agric. Biotechnol. 51, 102783. https://doi.org/10.1201/9781420077070

5.1 INTRODUCTION

In recent years, SSF has been used as an alternative to SmF for the production of value-added compounds and this process allows the direct use of agro-industrial by-products, reducing biocompound production costs at an industrial level and making the production process more environmentally sustainable due to the low consumption of water and energy (Abdul Manan and Webb, 2017). The increase of scale in SSF can be performed in several types of bioreactor designs. The simplest bioreactor design is a tray bioreactor, which usually belongs to the category of bioreactors without forced aeration and mixing events (Ashok et al., 2017). However, a problem that often arises during SSF, particularly during the scale increase, is the inefficient metabolic heat removal due to the low thermal conductivity of the solid materials used as substrates (Vauris et al., 2022). Furthermore, the temperature increase in the solid materials can impair microbial growth and degrade heat sensible compounds. Another challenge that must be addressed during scale up of SSF in static beds is the low gas transfer across the substrate, which can, once again, affect the growth of microorganisms and overall productivity (Ge et al., 2017). Thus, the design of more complex bioreactors, such as packet bed and horizontal drum bioreactors, with mixing and/or forced aeration is paramount to deal with these problems.

Filamentous fungi are widely applied in SSF due to their ability to grow in media with a low moisture content (Ramos-Sánchez et al., 2015). These microorganisms have been selected for the production of enzymes (Filipe et al., 2020; Oliveira et al., 2017; Sousa et al., 2022), organic acids (Dhillon et al., 2013; Torrado et al., 2011) and phenolic compounds (Dey and Kuhad, 2014). On the other hand, the utilization of yeasts in SSF is less abundant, however, in recent years, an increased number of studies reported their application in SSF for value-added compounds production as well as substrate biotransformation. *Yarrowia lipolytica* has been selected for erythritol production in SSF using agro-industrial and food wastes as substrates and scale up of the optimal conditions was successfully performed in 5 L flasks simulating a tray bioreactor (Liu et al., 2018, 2019). Nonetheless, the main biocompounds obtained by SSF with *Y. lipolytica* are lipases but these bioprocesses were mainly performed in small scale. For instance, this hydrolytic enzyme was produced by *Y. lipolytica* NCIM 3589 using, as solid substrates, palm kernel cake (Imandi et al., 2010), mustard oil cake (Imandi et al., 2013) and a mixture of sugarcane bagasse and wheat bran (Imandi and Garapati, 2007). In these studies, higher lipase production was achieved after medium supplementation with carbon and nitrogen sources. Other authors reported the utilization of by-products rich in fat as medium supplementation to induce lipase production by *Y. lipolytica* IMUFRJ 50682 (Farias et al., 2014; Rocha da Silva et al., 2019).

Regarding scale up of SSF for lipase production, Nascimento et al. (2021) studied the effect of medium supplementation in the production of lipase by *Y. lipolytica* IMUFRJ 50682 in a packed-bed bioreactor using 400 g of soybean hulls as a solid support. The authors reported a maximum lipase activity of 1350 U/L when soybean hulls were supplemented with yeast extract, bactopeptone and soybean oil. To our knowledge, there are no other studies reporting the scale up process of fermentations in solid state for the production of lipase by *Y. lipolytica.*

In the previous Chapter, several pretreatments were conducted to induce structural changes and increase sugars availability in the substrate mixture with equal parts of OC and SC. Although microwave irradiation resulted in higher lipase activity than the untreated mixture, the microwave pretreatment of large volumes of substrate is still challenging and, for this reason, scale-up of SSF for lipase production was performed without any pretreatment. In the present work, firstly the performance of *Y. lipolytica* W29 was compared to other strains of *Yarrowia* spp. to search for eventual the best lipase producers under SSF, and secondly, the scale up of the SSF process previously defined for lipase production by *Y. lipolytica* was executed using two types of bioreactors. In particular, trays were used with different substrate loadings and a horizontal drum with intermittent mixing was operated with and without forced aeration. Moreover, the impact of different bioreactor designs and oxygen availability on lipase and yeast cellular biomass production was studied.

5.2 MATERIALS AND METHODS

5.2.1 Raw materials

OC was acquired from Achsula SA, an olive mill production plant located in the Northern region of Portugal. This by-product results from olive oil extraction using a two-phase extraction system and was stored at -18 °C due to its high moisture content. SC was collected from a Portuguese vegetable oil production (Iberol SA) and, after milling, was stored at room temperature.

5.2.2 Microorganisms

In this work, strains of *Yarrowia* species from culture collections and isolates were used, such as *Y. lipolytica* W29 (ATCC 20460), *Y. lipolytica* NCYC 2904, *Y. lipolytica* NCYC 3535, *Y. lipolytica* MUCL 47034 and *Y. lipolytica* IMUFRJ 50862, isolated from an estuary in Rio de Janeiro (Hagler and Mendonça-

Hagler, 1981), *Y. lipolytica* JMY 3010, a W29 derivative strain with an overexpression in *LIP2* (Braga et al., 2015), *Y. divulgata* M445/4 and *Y. divulgata* 5257/2 both isolated from raw grounded beef (Nagy et al., 2013; Nagy, 2015). All strains were stored at - 80 °C in a solution containing 30 % (v/v) glycerol and cells were reactivated in YPDA (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 20 g/L) plates, which were stored at 4 °C. For inoculum preparation, yeast cells were collected from an agar plate and cultivated in 500 mL Erlenmeyer flasks with 100 mL of YPD (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L) medium. Flasks were kept overnight in an orbital incubator at 200 rpm and 27 °C.

5.2.3 SSF with *Yarrowia* strains

Small scale SSF was performed in 500 mL Erlenmeyer flasks with 10 g (dry mass) of a 50% (w/w, dry basis) mixture of OC and SC, the optimum substrate mixture for lipase production by *Y. lipolytica* W29 as described in Chapter 3. Flasks were autoclaved for 15 min at 121 °C and 2 mL of inoculum suspension (to attain 10^8 cells per gram of dry substrate) were added to the substrate mixture adjusting the final moisture content to 75% (w/w, wet basis). Flasks were kept in an incubator at 27 °C and after two days and enzyme extraction was performed as described in Chapter 3.

5.2.4 Scale up of SSF for lipase production

5.2.4.1 Tray bioreactors

SSF was performed in trays with different substrate loadings. 50 g (dry mass) of substrate mixture moistened with distilled water were added to a tray (16 x 11 x 5 cm) and sterilized for 15 min at 121 °C. After cooling, the solid substrate mixture was inoculated with 10 mL of inoculum suspension (3.8 mg of cells per g of dry substrate), increasing the moisture content to 75% (w/w, wet basis). The tray was sealed with a perforated plastic film to allow oxygen transfer and placed in an incubator at 27 °C for 2 days. For the experiments with higher substrate loading, a plastic container with 400 g (dry mass) of the substrate mixture moistened with distilled water was autoclaved in the conditions previously described. Moisture content was adjusted to 75% (w/w, wet basis) after adding 80 mL of the cellular suspension (3.8 mg of cells per g of dry substrate) and the inoculated substrate mixture was transferred to a tray (41 x 31 x 7 cm). Trays were covered with plastic film with and without small holes to assess the impact of oxygen availability on lipase production. Trays were kept in an incubator at 27 °C and the experiments were followed up to 4 days. For sampling, trays were moved to a laminar flow chamber and the substrate was

manually and aseptically mixed to ensure a homogenous sample collection. Enzyme extraction was performed as described in Chapter 3.

5.2.4.2 Horizontal drum bioreactor

The bioreactor (40 cm of length and 12 cm of internal diameter) used in these experiments was built in the author's own facilities and is schematically represented in Figure 5.1. It is made of Perspex acrylic composed of a horizontal cylindric drum with an internal volume of 4.5 L and includes a water jacket for temperature control. Paddles inside the bioreactor connected to a motor allowed substrate mixture at a rate of 2 rotations per minute. SSF was performed at two aeration rates of 0.1 and 0.2 L (at standard conditions of pressure at temperature)/min and also without forced aeration. In this case, oxygen was supplied by diffusion through cotton plugs placed at the sampling ports (Figure 5.1, numbers 3, 4 and 5). When forced aeration was employed, inlet air passed through a 0.22 μm filter followed by a humidification column filled with sterilized water. To start the SSF process, 200 g (dry mass) of the OC and SC mixture was sterilized and inoculated as previously described for trays experiments and substrate mixing was performed every 6 h for 15 min. To ensure homogenous sample collection, sampling was always performed at the three sampling ports (Figure 5.1, points 3, 4 and 5) and after substrate mixing. After sample collection, pH was measured in the solid substrate (Mettler Toledo LE427) and enzyme extraction was performed as described in Chapter 3.

5.2.5 Analytical methods

Moisture content of the substrate mixture was performed at the end of SSF to examine moisture loss during the fermentation process according to the standard methods from AOAC, (2005).

The aqueous extracts obtained after SSF were characterized regarding reducing sugars and soluble protein contents, cellular concentration and enzymatic activities of protease and lipase as described in Chapter 3. To test substrate specificity of the lipase produced in this work, lipase activity quantification was performed as described by Lopes et al. (2018) with some modifications: the substrates used in this spectrophotometric method were 4-nitrophenyl butyrate (4-NPB), 4-nitrophenyl laurate (4-NPL) or 4-nitrophenyl palmitate (4-NPP) at a concentration of 0.8 mM.

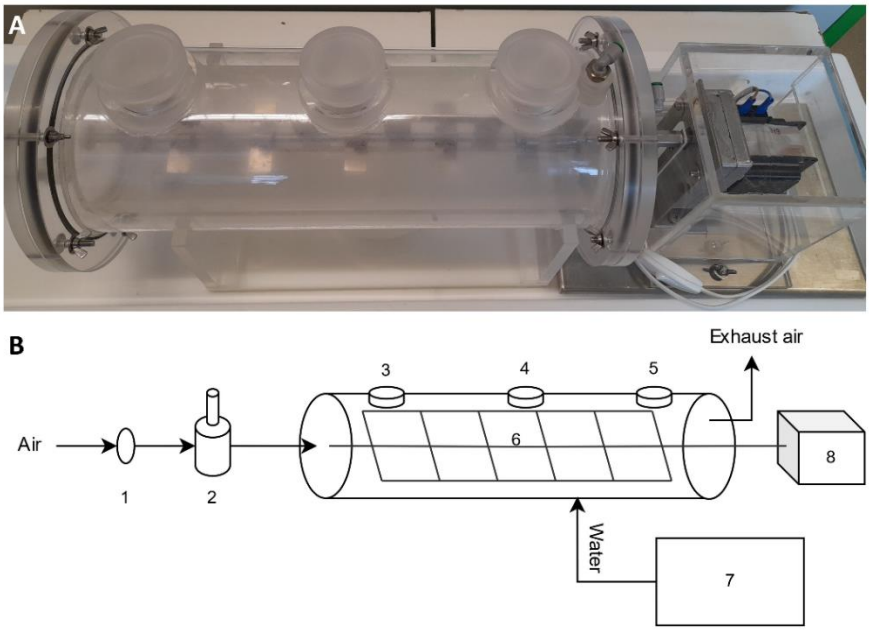

Figure 5.1: Horizontal drum bioreactor used in this work (A) and schematic representation of the system (B). 1: air filter; 2: humidification column; 3-5: sampling ports; 6: paddles; 7: water bath; 8: paddles motor.

5.2.6 Statistical analysis

The results are presented as mean ± standard deviation (SD) of two independent experiments.The experimental data were subjected to one-way analysis of variance (ANOVA) and Tukey's test for multiple comparison using GraphPad Prism. The analysis were performed with a confidence interval of 95%.

5.3 RESULTS

5.3.1 Screening of *Yarrowia* strains

In this Chapter, it was intended to compare the performance of the strain *Y. lipolytica* W29 with other strains of the same species and also with other strains from the new species *Yarrowia divulgata.* The production of polyols (Rakicka et al., 2016) and aromas (Braga et al., 2021) by *Y. divulgata* has been reported in submerged fermentation, showing the potential of its utilization in biotechnological processes for the production of value-added compounds.

Yarrowia strains were used in SSF with the selected substrate mixture to evaluate cellular growth, lipase and protease production in SSF for two days. The results obtained are shown in Figure 5.2. Even though all the microorganisms used in SSF are *Yarrowia* strains, high variability in terms of cellular growth and enzymes activities was observed after two days of SSF (Figure 5.2). Regarding *Y. lipolytica* strains, the lowest cellular concentration was attained for the strains NCYC 2904, W29 and JMY 3010, followed by MUCL 47034 and IMUFRJ 50682 (Figure 5.2A). The latter has been used in SSF processes using, as solid substrates, a mixture of OC and wheat bran (Lopes et al., 2016), canola (Souza et al., 2017), soybean and cottonseed cakes (Farias et al., 2014), demonstrating that this strain is well adapted to growth in solid substrates rich in lipids. However, the highest cellular density was observed with *Y. lipolytica* NCYC 3535. Additionally, these results revealed that fermentations in solid state represent suitable conditions for the growth of the two strains from *Y. divulgata,* with a highlight to the strain M445/4. The fact that these strains have been isolated from meat products (Nagy et al., 2013), could explain their capability to grow in solid substrates without or very low content of free water. To our knowledge this is the first report of the use of *Y. divulgata* in SSF processes.

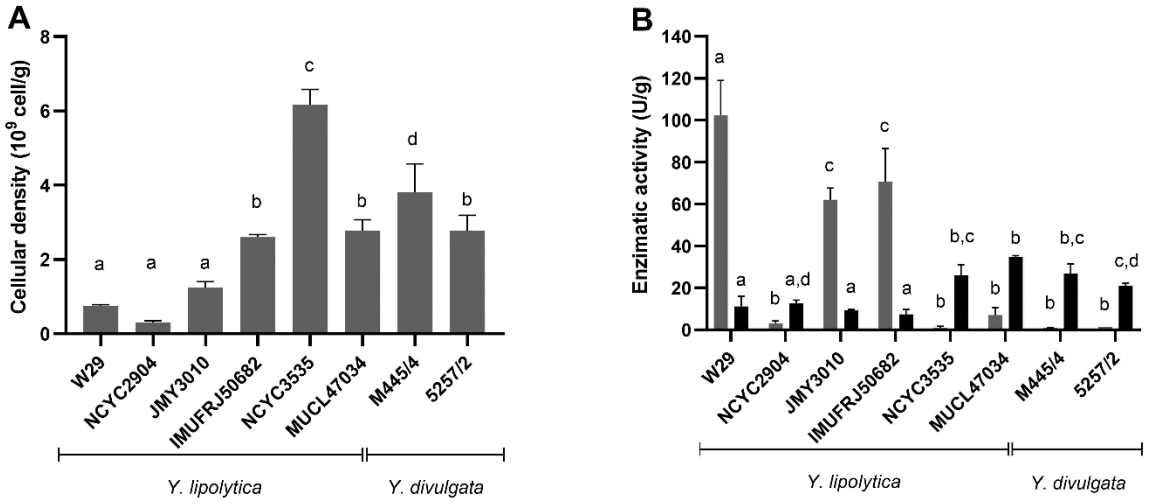

Figure 5.2: Cellular density (A) and enzymatic activities of lipase (grey bars) and protease (black bars) (B) obtained after two days of SSF with a 50 % (w/w, dry basis) mixture of OC and SC by different *Yarrowia* strains. The error bars represent the SD of two independent experiments. Bars with the same letter are not statistically different ($p > 0.05$). Statistical analysis was performed separately for each enzyme.

Despite the low cellular growth observed for *Y. lipolytica* W29, this strain produced the highest value of lipase activity (Figure 5.2B) followed by *Y. lipolytica* IMUFRJ 50682, which produced around 30% less lipase in the substrate mixture of OC and SC. Using the same strain, Farias et al., (2014) reported lower values of lipase activities than the herein obtained, 46 and 50 U/g, using soybean cake supplemented with soybean sludge and cottonseed cake, respectively. For this strain, the use of OC increased lipase production, since a mixture of OC and wheat bran led to a lipase activity of 94 U/g (Lopes et al., 2016). In the present study, lipase activity in the SSF extract obtained with *Y. lipolytica* W29, reached 100 U/g, showing the potential of using this strain for enzyme production in SSF with oil cakes as substrates without any medium supplementation. High lipase activity was also detected for *Y. lipolytica* JMY 3010, a strain with overexpression of *LIP2*, the gene that encodes the main extracellular lipase produced by *Y. lipolytica.* However, lipase activity was still below (60%) the obtained with strain W29, thus no advantages were found in the use of the genetically modified strain in SSF. Concerning the other strains used in the present study, lipase activity was low and below around 20% of the lipase produced by strain W29. Besides the low lipase production with *Y. lipolytica* NCYC 2904, this strain had the lowest cellular growth, in spite of not being statistically different than the observed with the strain W29, thus indicating that fermentations in solid state are not suitable for the growth of this strain, at least in the conditions employed in this work. A study using *Y. lipolytica* W29 and NCYC 2904 for microbial lipid production in submerged fermentation also reported a lower growth rate for *Y. lipolytica* NCYC 2904 in comparison to strain W29 (Pereira et al., 2023). Moreover, *Y. lipolytica* NCYC 3535 and MUCL 47034 and the two strains from *Y. divulgata* also presented low lipase activity after two days of SSF despite the high cellular growth attained. Figure 5.2B are also represented the results regarding protease activity. Besides lipase, *Y. lipolytica* is also able to secrete alkaline and acid proteases, being the proteases induced at alkaline pH the most secreted proteolytic enzyme (Ogrydziak, 2013). It is possible to observe that the strains NCYC 3535 and MUCL 47034 and *Y. divulgata* M445/4 and 5257/2 had higher protease production in comparison to *Y. lipolytica* W29, IMUFRJ 50682 and JMY 3010, the strains with the highest lipase activity detected. Several studies, both in solid state and submerged fermentations, reported that an abrupt decay on lipase activity is often followed by the increase in protease activity (Braga et al., 2012; Lopes et al., 2016; Moftah et al., 2013; Souza et al., 2017). The reason for this outcome is related to lipase degradation by proteolysis. Thus, the high proteolytic activity detected for the strains *Y. lipolytica* NCYC 3535 and MUCL 47034 and *Y. divulgata* M 445/4 and 5257/2 could explain the low lipase activity observed for these strains.

In summary, *Yarrowia* growth in SSF in the conditions used in this work appears to be strain dependent. This outcome could be related to the time that each strain requires to adapt to the solid matrix. Additionally, the metabolization of different compounds present in the substrate mixture could also explain these differences. For instance, *Y. lipolytica* NCYC 3535 is able to metabolize xylose, a sugar monomer that is scarcely consumed by *Y. lipolytica* W29 and is present in the hemicellulose fraction of the substrate mixture. Thus, the rate consumption of carbon and nitrogen sources may vary depending on the *Yarrowia* strain, resulting in different growth rates. Ultimately, regarding enzyme production, proteolytic activity appears to be the main cause for low lipase detection since higher protease activity was detected in the strains with low lipase activity. Taking into account the results of this screening, since highest lipase activity was detected with *Y. lipolytica* W29, the following scale-up experiments were performed with this strain.

5.3.2 SSF in tray bioreactors

Currently, several bioreactor designs are employed in the scale up of fermentations in solid state. As previously mentioned, tray bioreactors have static beds with very little or no agitation and are often operated without forced aeration, comprising one of the simplest bioreactors used in SSF (Arora et al., 2018; Ge et al., 2017). In the present work, scale up of the SSF process for lipase production with *Y. lipolytica* W29 with a mixture of 50% (w/w, dry basis) OC and SC was performed. Substrate loadings of 50 g and 400 g (dry basis) were used in tray bioreactors and, regardless of the substrate loading, bed height was kept at approximately 1 cm. The parameters analyzed in these experiments are presented in Table 5.1. The 5-fold increase of solid substrate mass from 10 g to 50 g did not affect any of the parameters analyzed in these experiments (Table 5.1). However, increasing the scale from 50 g to 400 g led to a significant decrease in cellular growth, corresponding to a reduction of 11% and 26% after two days of SSF for the system with perforated cover and the tray without cover perforations, respectively. The decrease in cellular growth was also accompanied by a significant reduction in sugar consumption, reaching a 40% reduction in the system without constant gas exchange. The experiments in trays with 400 g of substrate mixture covered with a plastic film with or without perforations were performed to examine the effect of oxygen availability on *Y. lipolytica* growth and enzyme production. One of the challenges in increasing scale in SSF in an open bioreactor, such as trays, is related to contamination. Likewise, the utilization of a sealed tray bioreactor could reduce the risk of contamination but, on the other hand, the low oxygen concentration in the gas in the system headspace may be detrimental to

microbial growth since *Y. lipolytica* is strictly aerobic (Gonçalves et al., 2014). In the present study, a decrease of 18% in cellular growth was observed in the system with a non-perforated cover, revealing that the low gas exchange, that occurred only during the manual substrate mixing, had a negative impact on *Y. lipolytica* growth.

Table 5.1: Cellular concentration, sugar consumption, lipase activity and specific activity obtained after SSF for two days in tray bioreactors with a 50 % (w/w, dry basis) mixture of OC and SC.

Parameters	**Substrate (g)**			
	10	**50**	**400***	**400#**
Cellular concentration (mg/g)	21 ± 1^{a}	19 ± 1^{a}	17 ± 1^{b}	14 ± 1^{c}
Sugar consumption (mg/g)	43 ± 1^{a}	42 ± 3^{a}	31 ± 5^{b}	24 ± 1^{c}
Lipase activity (U/g)	102 ± 17^{a}	$85 \pm 1^{a,b}$	$75 \pm 4^{b,c}$	56 ± 11^{c}
Lipase specific activity (U/mg)	3.9 ± 0.4^{a}	$5 \pm 1^{a,b}$	7 ± 2^{b}	4 ± 1^{a}

*System with perforated film

#System with unperforated film

Values represent the mean and SD of two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).

Regarding lipase activity, a decrease in the activity of this enzyme was observed with the scale increase. In particular, the scale up from 10 g to 50 g and from 10 g to 400 g, in trays with a perforated film, resulted in a 17% and 26% reduction in lipase activity, respectively. An important aspect to consider during SSF, particularly when using aerobic microorganisms, is the headspace of the bioreactors. In the present study, high lipase activity was attained in the smallest scale, which had a ratio of headspace volume to solid substrate mass of 50 cm^3/g. With the scale increase and the utilization of different bioreactors, a reduction in this ratio was observed, which could explain the decrease in lipase activity with the increase of solid loading. In particular, the increase of scale from 10 g to 50 g reduced the ratio between the headspace volume and solid substrate mass by 72% and a 62% reduction was observed with the 40-fold increase of solid loading. Mandari et al. (2020) observed a 23% reduction in lipase activity produced by *Aspergillus niger* MTCC 872 after scaling up from 10 g to 1 kg of a mixture of cottonseed cake, red gram husk and *Prosopis juliflora* in tray bioreactor with a 2.5 cm bed height. The authors concluded that the reduction in lipolytic activity in the higher SSF scale was related to the temperature used in the experiments, 35 °C, which led to moisture loss. However, in the present study, variations in

moisture content were very low, with moisture loss not exceeding 4%, possibly due to the selection of a lower temperature and bed height. Thus, another reason for the slight decrease in lipase activity with the increase in solid loading observed in this work, especially in trays with 400 g, could be linked with some heat and mass transfer limitations, that may arise at increasing scales. This leads to the formation of gas and temperature gradients throughout the solid beds, particularly in bioreactors without forced aeration and little to no agitation (Ge et al., 2017). While bed height employed in tray bioreactors can vary considerably, depending, for instance, on bioreactor configuration and operating conditions, low bed height is often used to avoid the decrease in oxygen availability and increase in temperature in the deeper layers of the bed. For instance, Rodríguez Couto et al. (2006) reported the use of a 1 cm bed for the production of laccase by *Trametes hirsuta* using grape seeds as solid substrate. Similarly, a mixture of soybean hulls and wheat bran spread in a 1 cm layer was the solid substrate selected to produce cellulosic enzymes by a co-culture of *Trichoderma reesei* (ATCC 26921) and *A. oryzae* (ATCC 12892) (Brijwani et al., 2010). Other authors reported that high bed height in trays can lead to poor microbial growth and low productivity. In particular, high lipase production by *A. niger* MTCC 872 in tray bioreactor was achieved with a bed height of 2.5 cm (Mandari et al., 2020). Increasing bed height to 3.5 and 4.5 cm led to a reduction in lipase activity. Moreover, Xie et al. (2013) studied the effect of substrate thickness on conidia production by *Beauveria bassiana* and reported that higher yield was obtained with a bed height of 2 cm. Increasing the bed height resulted in a reduction of conidia yield possibly related to the decrease of available oxygen and dissipation of metabolic heat. Considering the results obtained in this study and reports from other authors, it appears that, during the design and scale up of SSF with tray bioreactors, it is more advantageous to increase the number of trays, keeping low bed heights, in contrast to the use of fewer trays with high substrate loadings.

Despite the decrease in lipase production observed with the scale increase, high production was still achieved without the requirement for additional optimization steps. Additionally, an increase in lipase specific activity (units per mass of total soluble protein) was observed, particularly in trays with 400 g of substrate loading with a permeable cover that allowed oxygen diffusion (Table 5.1), in comparison to the smallest scale (10 g), where values of 3.9 U/mg were obtained. Although a fermented solid with high lipase activity can be used as a biocatalyst for esterification reactions (Rocha da Silva et al., 2019), the obtention of a liquid extract after SSF and further lipase purification can also be performed. In this case, the increase in lipase specific activity is of utmost importance to the downstream process of lipase purification.

To further understand the effect of oxygen availability on biocompound production by *Y. lipolytica*, SSF in trays with and without a perforated cover was followed up to four days and enzymatic activities of lipase and protease were monitored. Moreover, cell growth was also monitored and, by the end of SSF, yeast biomass was below 20 mg/g in both conditions tested. As can be observed in Figure 5.3, maximum lipase activity was detected on the second day of SSF regardless of the oxygen availability in the headspace. Nevertheless, as discussed above, values of maximum lipase activity obtained for the tray without a perforated cover are significantly lower in comparison to the tray with constant gas exchange (Table 5.1, Figure 5.3). After lipase activity peaked, a decrease was observed and, by the end of SSF, no lipase activity was detected when the tray was sealed with a perforated film. A similar enzymatic profile was obtained in SSF in smaller scale (Figure 3.3) as well as under SSF with canola cake using *Y. lipolytica* IMUFRJ 50682 (Souza et al., 2017). In contrast, lipase activity decrease in the tray with an unperforated cover was less abrupt and 50% of the activity of this enzyme was still detected on the fourth day of SSF. As previously described, the decrease in lipase activity could be a result of proteolysis since high protease production was observed at the end of fermentation, especially in the system with constant gas exchange. Protease activity observed in the system without a perforated film was 39% lower, which could explain the maintenance of lipase activity until the end of SSF.

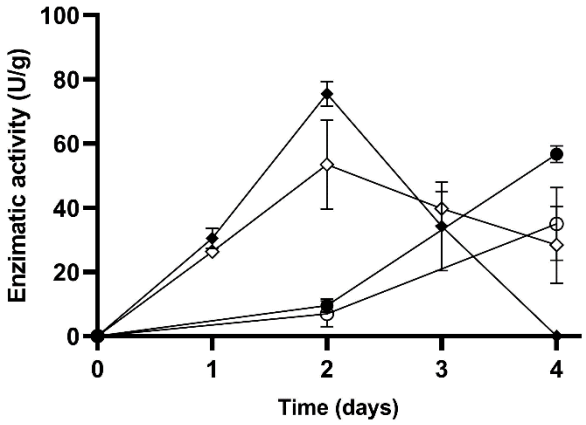

Figure 5.3: Time course of enzymatic activity of lipase (♦,◇) and protease (●,○) obtained during SSF with *Y. lipolytica* W29 for four days with 400 g (dry basis) of OC and SC in a tray-like bioreactor with a perforated film (filled symbols) and with an unperforated film (empty symbols). The error bars represent the SD of two independent experiments.

These results revealed that oxygen availability is fundamental for high lipase production and for microbial growth in SSF. The perforated cover used in tray bioreactors functions as the cotton plugs utilized to seal flasks in small scale SSF, both allowing constant gas exchange in the headspace. The fact that maximum lipase activity after the scale up from flasks with 10 g to trays with 400 g of substrate loading is still achieved in a short fermentation period is an advantage since it can minimize the risk of contamination.

5.3.3 SSF in horizontal drum bioreactor

Another type of bioreactor used in SSF processes is a horizontal drum bioreactor. This system is more complex in comparison with trays since paddles inside the bioreactor ensure substrate mixing, which can be continuous or intermittent (Soccol et al., 2017). Moreover, these bioreactors can operate without forced aeration or have forced aeration usually through the headspace. Forced aeration can be a significant aspect during SSF with aerobic microorganisms and its main goal is to provide oxygen to the microorganisms growth in the solid substrate as well as to remove carbon dioxide (Dhillon et al., 2013). According to Ge et al. (2017), the volume occupied by solid substrates in these bioreactors should be below 40% of the total drum volume to guarantee proper mixing. These experiments were performed with 200 g (dry basis) of substrate mixture which, together with the distilled water used to adjust the moisture content, occupied a fraction of the total volume of the bioreactor under 0.4. Additionally, the bioreactor was operated with and without forced aeration to examine the effect of this parameter on lipase activity and cellular growth. Samples were collected every 24 h and the results are shown in Figure 5.4.

When the horizontal drum bioreactor was operated with forced aeration, maximum cellular growth was achieved around the fourth day regardless of the air flow employed (Figure 5.4A). After this point, *Y. lipolytica* growth stabilized until the end of fermentation. In contrast, the absence of forced aeration in the horizontal drum bioreactor resulted in a slow growth rate in the first days of SSF. For instance, in the second day of fermentation, *Y. lipolytica* growth was 50% lower than the cellular concentration obtained for the bioreactor operated with forced aeration. Despite these results, cellular concentration increased until the end of SSF, reaching 100 mg of yeast biomass per gram of dry substrate mixture, corresponding to a 2-fold increase in yeast biomass in comparison to, for instance, the results attained in the horizontal drum operated at 0.2 L/min of airflow. Likewise, it appears that the forced aeration was important to reduce the lag phase of *Y. lipolytica* growth, increasing cellular growth at the beginning of SSF. However,

yeast growth stabilized earlier in these conditions, resulting in lower cellular concentration at the end of SSF.

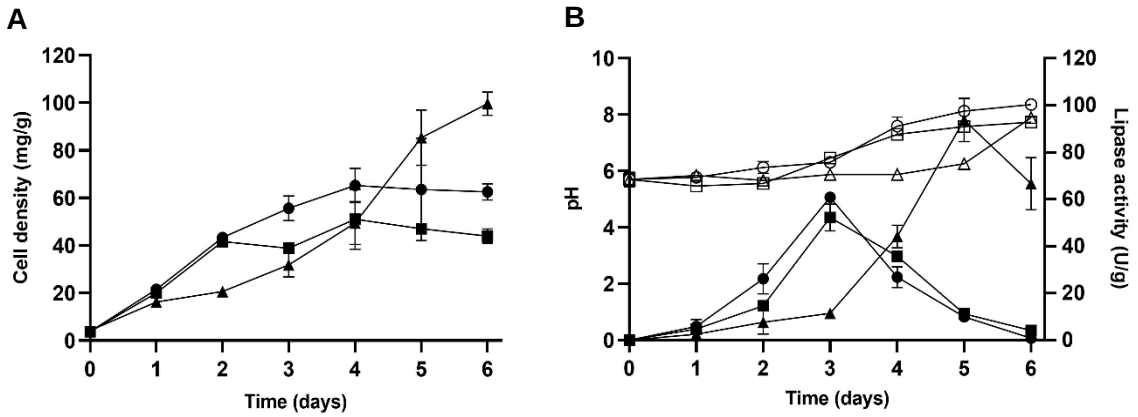

Figure 5.4: Time course of cellular density (A), lipase activity (filled symbols) and pH (empty symbols) (B) obtained during SSF with *Y. lipolytica* W29 for six days with 200 g (dry basis) of a 50 % (w/w, dry basis) mixture of OC and SC in a horizontal drum bioreactor with an airflow of 0.1 L/min (●,○) and 0.2 L/min (■,□) and without forced aeration (▲,△). The error bars represent the SD of two independent experiments.

Regardless of the airflow employed in the horizontal drum bioreactor, lipase activity peaked on the third day of cultivation (Figure 5.4B). Conversely, when air was supplied by diffusion through cotton plugs, this peak was detected on the fifth day of SSF and lipase activity increased around 64% in these conditions. Similarly to tray bioreactors, lipase activity decreased after reaching its maximum value and, when the bioreactor was operated with forced aeration, no lipase activity was detected by the end of SSF. At the beginning of *Y. lipolytica* growth, lipase is cell-bounded and the secretion of this lipolytic enzyme begins in the transition to the stationary phase (Pereira-Meirelles et al., 2000). Similarly, on SSF in small scale, maximum lipase activity was obtained when cellular growth was stabilized (Figure 3.3). Likewise, the fact that stabilization of yeast growth in the bioreactor with forced aeration occurred earlier in comparison to the bioreactor operated with air supplied by diffusion may justify the appearance of the lipase peak on the third day of SSF in contrast to the delayed maximum observed in the latter. In the first days of SSF, pH values were around 6 in all the aeration conditions. However, in the fermentations with forced aeration, pH started to increase in the fourth day, which was also the time point where the decrease in lipase activity was observed. On the contrary, in the bioreactor with aeration by diffusion, pH values were kept until the fifth day, where the pH measured was 6.3, similar to the pH observed when lipase

activity peaked in the forced aeration conditions. The decrease in lipase activity was also followed by an increase in the pH at the end of SSF, similar to what occurred in small scale SSF (Figure 3.3). Moreover, Lopes et al. (2016) and Souza et al. (2017) also reported an increase in pH following lipase maximum activity with *Y. lipolytica* IMUFRJ 50682 in SSF. pH increase in a fermentation medium is often linked with the release of proteases, which leads to amino acids deamination and ammonia release (Vargas et al., 2008). The higher growth rate observed at the beginning of SSF with forced aeration could also lead to the release of proteases to the extracellular medium earlier, resulting in lipase degradation and, consequently, a decline in lipase activity. Despite the differences regarding maximum lipase activity, similar lipase specific activity values were obtained in these experiments. In particular, values of 5.1 U/mg and 4.3 U/mg were obtained with an airflow of 0.1 L/min and 0.2 L/min, respectively. Moreover, when the bioreactor was operated without forced aeration lipase specific activity reached 4.1 U/mg. Although higher lipase activity was obtained in the horizontal drum with air supplied by diffusion (lowest aeration conditions), the delayed peak resulted in a decrease in lipase productivity (U/g/day) in these conditions. In particular, a 64% reduction in lipase productivity was observed compared to the results from small scale SSF (Figure 5.2B).

Despite their simplicity, tray bioreactors are associated with an intensive labor when manual mixing of the solid substrate is performed, which constitutes a drawback during the scale up of SSF at an industrial level. The risk of contamination and problems with heat and mass transfer in this system are often higher in comparison to other bioreactor designs (Soccol et al., 2017). Regardless of the energy employed in substrate agitation in the horizontal drum used in this work, one can consider that the energy costs are low since agitation was intermittent, being performed at specific time points throughout the SSF. *Yarrowia lipolytica* is a dimorphic microorganism, with transitions from yeast-to-hyphae growth depending on the environmental conditions (Timoumi et al., 2018). Thus, the formation of a mycelium is an advantage for its utilization in SSF processes, resulting in higher nutrient availability, microbial growth and production rates (Farias et al., 2014). While mixing events can have a negative impact on filamentous fungi growth due to damage in the fungal mycelia (Dhillon et al., 2013), the mechanical agitation performed in this work did not impair microbial growth, on the contrary, it had a positive effect on *Y. lipolytica* growth compared to tray bioreactors. In particular, as previously mentioned, after four days of SSF in trays, yeast biomass attained was below 20 mg/g and, in the horizontal drum bioreactor, yeast biomass detected at this time point was between 50 and 65 mg/g (Figure 5.4A). In fact, mixing in these bioreactors not only makes the process less labor intensive but also increases the surface area of the substrate exposed to air and improves heat dissipation (Arora et al., 2018). Comparing the results

regarding lipase production in the two different bioreactors tested in this work, it is possible to conclude that the use of trays, which are the simplest equipment, resulted in higher lipase productivity and specific activity. In contrast, a significant increase in cellular growth was observed with the horizontal drum bioreactor, especially when this bioreactor was operated without forced aeration. The improved microbial growth of *Y. lipolytica* W29 could be related to the substrate mixing, which could ensure higher metabolic heat dissipation and reduce the formation of gas gradients throughout the substrate bed.

5.3.4 Substrate specificity of lipases produced by SSF

The lipolytic substrate used to determine lipase activity in the present study was 4-NPB, a 4-nitrophenyl ester with a short carbon chain (C_4). Moreover, this ester derivative can be degraded not only by lipases but also by esterases. In fact, four genes encoding esterases are found in *Y. lipolytica* genome (Fickers et al., 2011). These carboxyl ester hydrolases are responsible for the formation and cleavage of ester bonds, usually on triglycerides with fatty acids with carbon chains smaller than 6 (Lopes et al., 2011). Thus, to test substrate specificity of the enzymes produced by *Y. lipolytica* W29 under SSF with a 50 % (w/w) mixture of OC and SC, 4-NPL (C_{12}) and 4-NPP (C_{16}) were also used as substrates in a spectrophotometric assay. Liquid extracts obtained from the fermented substrate mixture of the SSF process in tray with 50 g (dry basis) were used in these experiments.

Higher lipase activity was attained with 4-NPB, reaching (51 ± 1) U/g. Additionally, a reduction in lipase activity was observed with the other substrates employed in this experiment. In particular, a 65% and 59% lipase activity reduction was observed for 4-NPL and 4-NPP, respectively. Since lower lipolytic activity values were attained for these substrates, which are mainly hydrolyzed by lipases (Bunterngsook et al., 2010; Lopes et al., 2011), these results demonstrate that, besides lipases, esterases are also being produced during SSF with *Y. lipolytica* W29. Additionally, the lipase produced by *Y. lipolytica* in these conditions appears to have a slightly higher affinity towards long chain fatty acids since higher lipolytic activity was obtained for 4-NPP in comparison to 4-NPL. Oliveira et al. (2017) reported higher lipolytic activity using short chain fatty acids esters (C4 and C8) as substrates after SSF of oil cakes with *Aspergillus ibericus* MUM 03.49. Additionally, the fermented solid obtained was successfully used in esterification reactions to convert organic acids into aroma esters. Since the enzymes produced by *Y. lipolytica* W29 in this work also presented higher esterase activity, the use of enzymes produced by *Y. lipolytica* in SSF as a biocatalyst for the production of esters is a promising application for SSF with this oleaginous yeast. Despite the lower specificity towards long chain substrates observed in this study, the

enzymatic extract could also be applied in free fatty acids esterification during biodiesel production (Rocha da Silva et al., 2019). Moreover, the use of the remaining solid after enzyme extraction in animal feed formulations would lead to the complete valorization of OC and SC, promoting a circular economy.

5.4 Conclusions

The results obtained in this Chapter showed that SSF of OC mixed with SC is dependent on the *Yarrowia* strain since among the strains tested some are appropriate for lipase production, such as *Y. lipolytica* W29, while others for yeast biomass production, like *Y. lipolytica* NCYC 3535. Based on those differences, strain selection is of crucial relevance to the production of a target product, enzymes or microbial protein enrichment, for instance. Scale up of SSF for lipase production with *Y. lipolytica* W29 using a mixture of 50 % (w/w, dry basis) of OC and SC was effectively achieved in tray-like bioreactors and the oxygen availability and gas exchange was proven to be important factors to address in SSF operation. Horizontal drum bioreactor was, for the first time, applied in SSF of OC and SC with the yeast *Y. lipolytica* and proved to be a suitable alternative for increased scale SSF. The intermittent agitation used in this bioreactor had a positive effect on *Y. lipolytica* growth, resulting in a 5-fold increase in cellular concentration comparing the results from trays and the horizontal drum bioreactor operated without forced aeration. Thus, the use of oil cakes resultant from the production of olive and sunflower oils as solid substrates in the scale up of SSF proved to be a good strategy for enzyme production by *Y. lipolytica*.

5.5 References

Abdul Manan, M., Webb, C., 2017. Modern microbial solid state fermentation technology for future biorefineries for the production of added-value products. Biofuel Res. J. 4, 730–740. https://doi.org/10.18331/BRJ2017.4.4.5

AOAC, 18th editi. ed, 2005. , Official methods of analysis of the Association of Official Analytical Chemists. Washington DC, USA.

Arora, S., Rani, R., Ghosh, S., 2018. Bioreactors in solid state fermentation technology: Design, applications and engineering aspects. J. Biotechnol. 269, 16–34. https://doi.org/10.1016/j.jbiotec.2018.01.010

Ashok, A., Doriya, K., Rao, D.R.M., Kumar, D.S., 2017. Design of solid state bioreactor for industrial applications: An overview to conventional bioreactors. Biocatal. Agric. Biotechnol. 9, 11–18. https://doi.org/10.1016/j.bcab.2016.10.014

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 7, 248–254.

Braga, A., Crutz-Le Coq, A.M., Dulermo, R., Nicaud, J.M., Belo, I., 2015. Effect of *POX* genotype and Lip2p overexpression on lactone production and reconsumption by *Yarrowia lipolytica* using castor oil as substrate. Process Biochem. 50, 1357–1362. https://doi.org/10.1016/j.procbio.2015.05.019

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. J. Chem. Technol. Biotechnol. 96, 2940–2949. https://doi.org/10.1002/jctb.6849

Braga, A., Gomes, N., Belo, I., 2012. Lipase induction in *Yarrowia lipolytica* for castor oil hydrolysis and its effect on γ-decalactone production. J. Am. Oil Chem. Soc. 89, 1041–1047. https://doi.org/10.1007/s11746-011-1987-5

Brijwani, K., Oberoi, H.S., Vadlani, P. V., 2010. Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. Process Biochem. 45, 120–128. https://doi.org/10.1016/j.procbio.2009.08.015

Bunterngsook, B., Kanokratana, P., Thongaram, T., Tanapongpipat, S., Uengwetwanit, T., Rachdawong, S., Vichitsoonthonkul, T., Eurwilaichitr, L., 2010. Identification and characterization of lipolytic enzymes from a peat-swamp forest soil metagenome. Biosci. Biotechnol. Biochem. 74, 1848–1854. https://doi.org/10.1271/bbb.100249

Christoforou, E., Fokaides, P.A., 2016. A review of olive mill solid wastes to energy utilization techniques. Waste Manag. 49, 346–363. https://doi.org/10.1016/j.wasman.2016.01.012

Costa, A.R., Salgado, J.M., Lopes, M., Belo, I., 2022. Valorization of by-products from vegetable oil industries: Enzymes production by *Yarrowia lipolytica* through solid state fermentation. Front. Sustain. Food Syst. 6. https://doi.org/10.3389/fsufs.2022.1006467

Dey, T.B., Kuhad, R.C., 2014. Enhanced production and extraction of phenolic compounds from wheat

by solid-state fermentation with Rhizopus oryzae RCK2012. Biotechnol. Reports 4, 120–127. https://doi.org/10.1016/j.btre.2014.09.006

Dhillon, G.S., Brar, S.K., Kaur, S., Verma, M., 2013. Bioproduction and extraction optimization of citric acid from *Aspergillus niger* by rotating drum type solid-state bioreactor. Ind. Crops Prod. 41, 78–84. https://doi.org/10.1016/j.indcrop.2012.04.001

Donner, M., Radić, I., Erraach, Y., El Hadad-Gauthier, F., 2022. Implementation of Circular Business Models for Olive Oil Waste and By-Product Valorization. Resources 11, 1–18. https://doi.org/10.3390/resources11070068

Farias, M.A., Valoni, E.A., Castro, A.M., Coelho, M.A.Z., 2014. Lipase production by *Yarrowia lipolytica* in solid state fermentation using different agro industrial residues. Chem. Eng. Trans. 38, 301–306. https://doi.org/10.3303/CET1438051

Fickers, P., Marty, A., Nicaud, J.M., 2011. The lipases from *Yarrowia lipolytica*: Genetics, production, regulation, biochemical characterization and biotechnological applications. Biotechnol. Adv. 29, 632–644. https://doi.org/10.1016/j.biotechadv.2011.04.005

Filipe, D., Fernandes, H., Castro, C., Peres, H., Oliva-Teles, A., Belo, I., Salgado, J.M., 2020. Improved lignocellulolytic enzyme production and antioxidant extraction using solid-state fermentation of olive pomace mixed with winery waste. Biofuels, Bioprod. Biorefining 14, 78–91. https://doi.org/10.1002/bbb.2073

Ge, X., Vasco-Correa, J., Li, Y., 2017. Solid-State Fermentation Bioreactors and Fundamentals, in: Current Developments in Biotechnology and Bioengineering: Bioprocesses, Bioreactors and Controls. pp. 381–402. https://doi.org/10.1016/B978-0-444-63663-8.00013-6

Gonçalves, F.A.G., Colen, G., Takahashi, J.A., 2014. *Yarrowia lipolytica* and Its Multiple Applications in the Biotechnological Industry. Sci. World J. 1–14. https://doi.org/10.1155/2014/476207

Hagler, A.N., Mendonça-Hagler, L.C., 1981. Yeasts from Marine and Estuarine Waters with Different Levels of Pollution in the State of Rio de Janeiro, Brazil. Appl. Environ. Microbiol. 41, 173–178. https://doi.org/10.1128/aem.41.1.173-178.1981

Imandi, S.B., Garapati, H.R., 2007. Lipase Production by *Yarrowia lipolytica* NCIM 3589 in Solid State Fermentation Using Mixed Substrate. Res. J. Microbiol. https://doi.org/10.3923/jm.2007.469.474

Imandi, S.B., Karanam, S.K., Garapati, H.R., 2013. Use of Plackett-Burman design for rapid screening of nitrogen and carbon sources for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from mustard oil cake (Brassica napus). Brazilian J. Microbiol. 44, 915–921. https://doi.org/10.1590/S1517-83822013005000068

Imandi, S.B., Karanam, S.K., Garapati, H.R., 2010. Optimization of media constituents for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from palm Kernal cake (Elaeis guineensis). Adv. Biosci. Biotechnol. 1, 115–121. https://doi.org/10.4236/abb.2010.12016

Liu, X., Yan, Y., Zhao, P., Song, J., Yu, X., Wang, Z., Xia, J., Wang, X., 2019. Oil crop wastes as substrate candidates for enhancing erythritol production by modified *Yarrowia lipolytica* via one-step solid state

fermentation. Bioresour. Technol. 294, 122194. https://doi.org/10.1016/j.biortech.2019.122194

Liu, X., Yu, X., Zhang, T., Wang, Z., Xu, Jiaxing, Xia, J., He, A., Yan, Y., Xu, Jiming, 2018. Novel two-stage solid-state fermentation for erythritol production on okara–buckwheat husk medium. Bioresour. Technol. 266, 439–446. https://doi.org/10.1016/j.biortech.2018.07.009

Lopes, D.B., Fraga, L.P., Fleuri, L.F., Macedo, G.A., 2011. Lipase and esterase - to what extent can this classification be applied accurately? Cienc. e Tecnol. Aliment. 31, 608–613. https://doi.org/10.1590/s0101-20612011000300009

Lopes, M., Miranda, S.M., Alves, J.M., Pereira, A.S., Belo, I., 2018. Waste Cooking Oils as Feedstock for Lipase and Lipid-Rich Biomass Production. Eur. J. Lipid Sci. Technol. 1–9. https://doi.org/10.1002/ejlt.201800188

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. Crit. Rev. Biotechnol. 42, 163–183. https://doi.org/10.1080/07388551.2021.1931016

Lopes, V.R.O., Farias, M.A., Belo, I.M.P., Coelho, M.A.Z., 2016. Nitrogen sources on TPOMW valorization through solid state fermentation performed by *Yarrowia lipolytica*. Brazilian J. Chem. Eng. 33, 261–270. https://doi.org/10.1590/0104-6632.20160332s20150146

Mandari, V., Nema, A., Devarai, S.K., 2020. Sequential optimization and large scale production of lipase using tri-substrate mixture from *Aspergillus niger* MTCC 872 by solid state fermentation. Process Biochem. 89, 46–54. https://doi.org/10.1016/j.procbio.2019.10.026

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426–428.

Moftah, O.A.S., Grbavčić, S., Žuža, M., Luković, N., Bezbradica, D., Knežević-Jugović, Z., 2012. Adding value to the oil cake as a waste from oil processing industry: Production of lipase and protease by *Candida utilis* in solid state fermentation. Appl. Biochem. Biotechnol. 166, 348–364. https://doi.org/10.1007/s12010-011-9429-2

Moftah, O.A.S., Grbavcic, S.Z., Moftah, W.A.S., Lukovic, N.D., Prodanovic, O.L., Jakovetic, S.M., Kneževic-Jugovic, Z.D., 2013. Lipase production by *Yarrowia lipolytica* using olive oil processing wastes as substrates. J. Serbian Chem. Soc. 78, 781–794. https://doi.org/10.2298/JSC120905005M

Nagy, E., Niss, M., Dlauchy, D., Arneborg, N., Nielsen, D.S., Péter, G., 2013. *Yarrowia divulgata* f.a., sp. nov., a yeast species from animal-related and marine sources. Int. J. Syst. Evol. Microbiol. 63, 4818–4823. https://doi.org/10.1099/ijs.0.057208-0

Nagy, E.S., 2015. Biodiversity of food spoilage Yarrowia group in different kinds of food. Corvinus University of Budapest, Hungary.

Nascimento, F.V. de, de Castro, A.M., Secchi, A.R., Coelho, M.A.Z., 2021. Insights into media supplementation in solid-state fermentation of soybean hulls by *Yarrowia lipolytica*: Impact on lipase production in tray and insulated packed-bed bioreactors. Biochem. Eng. J. 166, 107866. https://doi.org/10.1016/j.bej.2020.107866

Ogrydziak, D., 2013. Acid and Alkaline Extracellular Proteases of *Yarrowia lipolytica*, in: Barth G. (Eds) Yarrowia Lipolytica. Microbiology Monographs. https://doi.org/10.1007/978-3-642-38583-4

Oliveira, F., Souza, C.E., Peclat, V.R.O.L., Salgado, J.M., Ribeiro, B.D., Coelho, M.A.Z., Venâncio, A., Belo, I., 2017. Optimization of lipase production by *Aspergillus ibericus* from oil cakes and its application in esterification reactions. Food Bioprod. Process. 102, 268–277. https://doi.org/10.1016/j.fbp.2017.01.007

Pereira-Meirelles, F. V., Rocha-Leão, M.H.M., Sant'Anna, G.L., 2000. Lipase location in *Yarrowia lipolytica* cells. Biotechnol. Lett. 22, 71–75. https://doi.org/10.1023/A:1005672731818

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. Renew. Energy 202, 1470–1478. https://doi.org/10.1016/j.renene.2022.12.012

Rakicka, M., Kieroń, A., Hapeta, P., Neuvéglise, C., Lazar, Z., 2016. Sweet and sour potential of yeast from the *Yarrowia* clade. Biomass and Bioenergy 92, 48–54. https://doi.org/10.1016/j.biombioe.2016.06.004

Ramos-Sánchez, L.B., Cujilema-Quitio, M.C., Julian-Ricardo, M.C., Cordova, J., Fickers, P., 2015. Fungal Lipase Production by Solid-State Fermentation. J. Bioprocess. Biotech. 5, 105–116. https://doi.org/10.1007/978-1-59259-991-2_10

Rocha da Silva, J., de Souza, C.E.C., Valoni, E., de Castro, A.M., Coelho, M.A.Z., Ribeiro, B.D., Henriques, C.A., Langone, M.A.P., 2019. Biocatalytic esterification of fatty acids using a low-cost fermented solid from solid-state fermentation with *Yarrowia lipolytica*. 3 Biotech 9, 38. https://doi.org/10.1007/s13205-018-1550-2

Rodríguez Couto, S., López, E., Sanromán, M.Á., 2006. Utilisation of grape seeds for laccase production in solid-state fermentors. J. Food Eng. 74, 263–267. https://doi.org/10.1016/j.jfoodeng.2005.03.004

Salgado, J.M., Abrunhosa, L., Venâncio, A., Domínguez, J.M., Belo, I., 2014. Integrated use of residues from olive mill and winery for lipase production by solid state fermentation with *Aspergillus* sp. Appl. Biochem. Biotechnol. 172, 1832–1845. https://doi.org/10.1007/s12010-013-0613-4

Soccol, C.R., Costa, E.S.F., Letti, L.A.J., Karp, S.G., Vandenberghe, L.P. de S., Woiciechowski, A.L., 2017. Recent developments and innovations in solid state fermentation. Biotechnol. Res. Innov. 1, 52–71. https://doi.org/10.1016/j.biori.2017.01.002

Sousa, D., Salgado, J.M., Cambra-López, M., Dias, A.C.P., Belo, I., 2022. Degradation of lignocellulosic matrix of oilseed cakes by solid-state fermentation: fungi screening for enzymes production and antioxidants release. J. Sci. Food Agric. 102, 1550–1560. https://doi.org/10.1002/jsfa.11490

Souza, C., Farias, M.A., Ribeiro, B.D., Coelho, M.A.Z., 2017. Adding Value to Agro-industrial Co-products from Canola and Soybean Oil Extraction Through Lipase Production Using *Yarrowia lipolytica* in Solid-State Fermentation. Waste and Biomass Valorization 8, 1163–1176.

Timoumi, A., Guillouet, S.E., 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

Torrado, A.M., Cortés, S., Salgado, J.M., Max, B., Rodríguez, N., Bibbins, B.P., Converti, A., Domínguez, J.M., 2011. Citric acid production from orange peel wastes by solid-state fermentation. Brazilian J. Microbiol. 42, 394–409. https://doi.org/10.1590/S1517-83822011000100049

Vargas, G.D.L.P., Treichel, H., de Oliveira, D., Beneti, S.C., Freire, D.M.G., Di Luccio, M., 2008. Optimization of lipase production by *Penicillium simplicissimum* in soybean meal. J. Chem. Technol. Biotechnol. 83, 47–54. https://doi.org/10.1002/jctb

Vauris, A., Valcauda, S., Husson, F., Coninck, J. De, 2022. A novel method to assess heat transfer and impact of relevant physicochemical parameters for the scaling up of solid state fermentation systems. Biotechnol. Reports 36. https://doi.org/10.1016/j.btre.2022.e00764

Vong, W.C., Au Yang, K.L.C., 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

Vong, W.C., Hua, X.Y., Liu, S.Q., 2018. Solid-state fermentation with *Rhizopus oligosporus* and *Yarrowia lipolytica* improved nutritional and flavour properties of okara. LWT - Food Sci. Technol. 90, 316–322. https://doi.org/10.1016/j.lwt.2017.12.050

Xie, L., Chen, H., Yang, J., 2013. Conidia production by *Beauveria bassiana* on rice in solid-state fermentation using tray bioreactor. Adv. Mater. Res. 610–613, 3478–3482. https://doi.org/10.4028/www.scientific.net/AMR.610-613.3478

Yano, Y., Oikawa, H., Satomi, M., 2008. Reduction of lipids in fish meal prepared from fish waste by a yeast *Yarrowia lipolytica*. Int. J. Food Microbiol. 121, 302–307. https://doi.org/10.1016/j.ijfoodmicro.2007.11.012

6 PRODUCTION OF *Y. LIPOLYTICA* W29 BIOMASS FROM OLIVE CAKE HYDROLYSATES

The challenges involved in OC generation and handling in some Mediterranean countries, including Portugal, makes it imperative to examine several strategies for the valorization of this by-product. Besides SSF, pretreatments can also be performed in these types of materials, originating a hydrolysate with high content of sugars that can be used as culture medium in SmF processes. Due to its recalcitrant nature, before enzymatic hydrolysis, OC must undergo a pretreatment that induces changes in its structure, resulting in higher cellulose and hemicellulose degradation and sugars release. In the present Chapter, ionic liquids and hydrothermal pretreatments were the selected methods to pretreat OC. The remaining solids were then submitted to enzymatic hydrolysis and the obtained hydrolysates were used as culture medium for biomass production by *Y. lipolytica* W29. Higher delignification was observed with ionic liquids pretreatment and, in contrast, hydrothermal pretreatment at 210 °C resulted in a solid with higher glucan content. The sugars present in the OC hydrolysates based medium were effectively metabolized by *Y. lipolytica*, including xylose. Moreover, the yeast biomass obtained in these conditions was rich in protein and, when olive oil was supplemented to the medium, also lipids.

6.1 INTRODUCTION

Olive oil production is a relevant activity in several European countries and the production of this vegetable oil reached, in these countries, around 2,271,700 t in the crop year of 2021/22 (European Comission, 2023). Olive oil extraction using the two-phase centrifugation system leads to the production of a semi-solid material named two-phase olive mill waste or simply OC. Although several biotechnological approaches for OC treatment and valorization have been proposed, such as SSF, OC high content of phenolic compounds and lack of nitrogen usually have a negative impact on microbial growth. Thus, mixture with other agro-industrial by-products (Costa et al., 2022; Filipe et al., 2020; Lopes et al., 2016) or pretreatments (Moftah et al., 2013, 2012) are required to improve overall productivity of SSF processes of OC. Another strategy that could be employed in the valorization of this underexplored by-product involves enzymatic hydrolysis of its polysaccharide' fraction, resulting in the release of monomeric sugars, which then could be used for microbial growth and value-added compound production. However, OC is characterized by a high content of lignin, reaching values above 50 % (w/w, dry mass) in some cases (Salgado et al., 2014), and its presence in lignocellulosic biomass can function as a barrier protecting cellulose from enzymatic degradation. The recalcitrant nature of lignocellulosic biomass can be overcome by physical and chemical pretreatments of these materials, which can improve digestibility and the efficiency of enzymatic hydrolysis (Melati et al., 2019).

Hydrothermal pretreatment is a low cost method used for lignocellulosic biomass fractioning and only requires the use of water at high temperatures, ranging from 140 and 220 °C (Pu et al., 2013). This method can reduce the recalcitrant nature of lignocellulosic biomass, resulting in the solubilization of the hemicellulosic fraction while altering the structure and chemical composition of lignin, which mostly remains in the solid phase after the pretreatment (Kellock et al., 2019). Hydrothermal pretreatment has been selected for the processing of by-products from olive oil industries such as olive tree pruning (Romero-García et al., 2022), olive stones (Cuevas et al., 2015; Miranda et al., 2019) and OC (Freitas et al., 2022; Manzanares et al., 2020; Miranda et al., 2019). In contrast, some pretreatments resort to chemicals to alter the recalcitrant nature of lignocellulosic biomass, including acids, alkalis, organic solvents and ionic liquids (ILs) (Wagle et al., 2022). The latter are defined as salts with melting points below 100 °C and, generally, are characterized by a high thermal and chemical stability, low conductivity and vapor pressure and high viscosity (Hayes et al., 2015; Usmani et al., 2020). Although pretreatment with dialkylimidazolium-based ILs have shown promising results in lignocellulosic biomass fractioning, problems related to high costs, high viscosity and low biocompatibility led to the development of ILs with improved properties (Zhang et al., 2021). In this context, cholinium-based ILs are an eco-friendly and

biocompatible alternative that can be synthesized using renewable compounds and amino acids (Le Donne and Bodo, 2021).

Regardless of the method employed for lignocellulosic biomass pretreatment, and after enzymatic hydrolysis of the pretreated material, the obtained hydrolysate rich in sugars can be used as culture medium for the growth of microorganisms and biocompound production. The non-conventional yeast *Y. lipolytica* is known for its ability to convert several substrates into value-added compounds (Lopes et al., 2022) and represents a good candidate for SmF using hydrolysates from lignocellulosic biomass, due to its resistance to inhibitory compounds (Dias et al., 2023). Moreover, this oleaginous yeast has the GRAS status and its biomass is safe to be employed in feed formulations for animals and human consumption (Groenewald et al., 2014).

In the present Chapter, the impact of hydrothermal and ILs pretreatment in the chemical composition of OC was assessed. The potential of the hydrolysate obtained after enzymatic hydrolysis of the remaining solids as culture medium for the growth of *Y. lipolytica* W29 was evaluated and the characterization of the yeast biomass obtained was performed.

6.2 MATERIALS AND METHODS

6.2.1 Microorganism

Yarrowia lipolytica W29 (ATCC 20460) was stored in a 30 % (v/v) glycerol solution at - 80 °C and revived in YPDA medium (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 20 g/L). Inoculum was prepared with yeasts cells collected from an agar plate and cultivated in 100 mL of YPD medium (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L). Erlenmeyer flasks were kept in an orbital incubator overnight at 200 rpm and 27 °C.

6.2.2 Raw material and solids characterization

OC obtained from olive oil extraction in a two-phase system was collected from an olive mill in the northern region of Portugal (Achsula SA) and stored at - 18 °C due to its high moisture content. OC was characterized in terms of lignin and main polysaccharides as described by Leite et al. (2016). The solids recovered after the pretreatments were also characterized using the same methods.

6.2.3 Hydrothermal pretreatment

Hydrothermal pretreatment was carried using a stainless steel reactor (Parr Instruments, Moline, IL) with 50 g (dry mass) of OC mixed with distilled water at a solid loading of 10% (w/w). Agitation was kept at 120 rpm and experiments were carried until the temperature reached 190 °C or 210 °C. In the first case, bioreactor cool down was performed after reaching the target temperature and in the latter maximum temperature of 210 °C was maintained for 5 min. After this point, the reactor was cooled down until 50 °C and the solid and the liquid were separated by filtration through a fine mesh net followed by centrifugation at 8000 rpm for 10 min. The remaining solid was weighted and its moisture content was determined according to the methodology from AOAC (2005) to calculate the solid recovered after pretreatment. Part of the remaining solid was washed with distilled water, dried at 60 °C for 24 h and characterization was performed as previously described. For each experiment, the severity factor (log R_0) was calculated from the value of R_0 according to equation 1 (Ruiz et al., 2017):

$$R_0 = \int_0^t \exp\left(\frac{T-100}{14.75}\right) dt \quad (1)$$

Where the temperature (T, °C) is a function of time (t, min), 100 °C is the reference temperature and 14.75 °C is a constant related with the normal energy of activation.

The solubilization of glucan, xylan and lignin was calculated as follows:

$$Solubilization\ (\%) = \left(1 - \frac{Solid\ yield \times content\ in\ pretreated\ OC}{content\ in\ raw\ OC}\right) \times 100 \quad (2)$$

6.2.4 ILs pretreatment

The IL used in this work was cholinium glycinate [N1112OH][Gly], which was synthesized as described by Deive et al. (2015). The pretreatment of OC was performed following the methods reported by Outeiriño et al. (2019). Briefly, 0.5 g of dry OC were treated with 10 g (5% w/w) of [N1112OH][Gly] in a 100 mL glass bottle, which was placed into a sand bath heated on a hot plate with magnetic stirring. The pretreatment was performed at 90 °C for 2 h, 4 h, 8 h and 16 h or at 110 °C for 2 h and 4 h. Subsequently, precipitation of the carbohydrate-rich material was performed by adding 50 mL of an acetone/water (1:1 v/v) solution to the mixture, which was stirred at room temperature for 30 min. This step was followed by centrifugation at 2755 *g* for 30 min and the material rich in carbohydrates was

separated from the supernatant (with high content in lignin) by filtration with a nylon filter. 40 mL of water was added to the remaining solid to ensure the ionic liquid and acetone removal. This step was performed 4 times and solid recovery was achieved by centrifugation using the conditions previously described. Then, the pretreated OC was oven dried at 30 °C for 24 h and was gravimetrically measured and characterized as described above. After selection of the optimum pretreatment conditions, 5 g of OC were pretreated for 16 h at 90 °C and the following steps were performed was previously described. The recovery of ILs was performed by mixing the IL containing streams in a Büchi rotavapor R-215 at 50 °C with variable pressure (from 100 to 40 mbar).

Glucan, xylan and lignin solubilization was calculated as depicted in equation 2.

6.2.5 Enzymatic hydrolysis

Solids resultant from ILs and hydrothermal pretreatments were submitted to enzymatic hydrolysis using the enzymatic cocktail Cellic Ctec2 (Sigma) with a cellulase (CMCases) concentration of 100 U/g. The pretreated solids were mixed with the enzymatic cocktail diluted in 0.05 M sodium citrate buffer (pH 4.8) with a solid loading of 10% (w/w) and the flasks were placed in an orbital incubator for 72 h at 50 °C. Enzymatic hydrolysis in the same conditions was performed with raw OC as a control assay. After enzymatic hydrolysis the solid and the hydrolysate were separated by vacuum filtration and a sample of the liquid was analyzed in HPLC as described below. The remaining hydrolysate was stored at - 18 °C until its utilization in SmF and microplate assays. Saccharification of the solids, which determines enzymatic hydrolysis efficiency, was expressed as a percentage of the maximum theoretical yield of glucose or xylose that could be released if glucan or xylan in OC were completely hydrolyzed. This calculation was performed was follows:

$$Saccharification\ (\%) = \frac{Sugar\ in\ hydrolysate\ (g/L)}{Sugar\ in\ OC\ (g/L)} \times 100 \quad (3)$$

Sugar concentration is the beginning of the enzymatic hydrolysis was taken into account in this calculation to avoid overestimation of sugar release by the action of the enzymatic cocktail.

6.2.6 Fermentation in microplate assays

Experiments in 96-well microplates were performed to access the growth of *Y. lipolytica* W29 in the hydrolysate obtained after enzymatic hydrolysis of the OC pretreated with ILs. Yeast culture grown overnight in YPD was centrifuged at 5000 rpm for 10 min and the pellet was resuspended in 0.9 % (w/v) NaCl. Each well was filled with 30 μL of yeast suspension to attain an optical density (OD, λ = 600 nm) of 0.5, 240 μL of OC hydrolysate and 30 μL of supplements at three different conditions: 1 – addition of 6.7 g/L YNB (without amino acids and ammonium sulfate); 2 – addition of 2 g/L corn steep liquor (CSL) and 5 g/L ammonium sulphate and 3 – addition of 4 g/L CSL and 5 g/L ammonium sulphate. As control experiments, OC hydrolysate was replaced by 240 μL of 8 g/L glucose. In both conditions, medium pH at the start of the experiments was adjusted to 7.2. Microplates were placed in an orbital incubator at 27 °C and 150 rpm for 33 h and OD (λ = 600 nm) was measured at the beginning and at the end of incubation. OD variation was calculated by subtracting the OD value obtained at the beginning to the value registered after 33 h of incubation.

6.2.7 Erlenmeyer flasks batch cultures

A batch experiment with OC hydrolysate from OC pretreated with ILs was performed with 5 mL of hydrolysate in a 25 mL Erlenmeyer flask. 2 g/L CSL and 5 g/L ammonium sulphate were used was medium supplementation. The pH was adjusted to 7.2 with NaOH 2 M at the beginning of the experiments and samples were collected at specific time points. Batch experiments with OC hydrolysate obtained after hydrothermal pretreatment were carried out in Erlenmeyer flasks of 250 mL with a volume of 50 mL and OC hydrolysate based medium was supplemented with 2 g/L CSL and 5 g/L ammonium sulfate. Supplementation was also performed with 7 g/L olive oil and 5 g/L arabic gum. The pH of cultures with OC hydrolysate obtained after hydrothermal pretreatment was adjusted to 7.2 by the addition of HCl 2 M or NaOH 2 M in the beginning of the cultivation and at each sample collection. In both experiments, the inoculum was centrifuged as previously described and yeast cells were resuspended in the culture medium attaining a biomass concentration of 0.5 g/L. Flasks were kept in an orbital incubation at 200 rpm and 27 °C for 33 h and 48 h in cultures with OC hydrolysate obtained from ILs and hydrothermal pretreatments, respectively.

6.2.8 Analytical methods

Glucose, xylose, acetic and formic acids, 5-HMF and furfural present in the hydrolysates after enzymatic hydrolysis were determined by high-performance liquid chromatography (HPLC, LC 2060C, Shimadzu, Japan) as described in Chapter 4.

Samples from batch cultures were collected at specific time points for quantification of biomass, reducing sugars, enzymes activities, microbial lipids and crude protein.

Biomass concentration was determined by measuring OD (λ = 600 nm) or, in the case of cultures with the hydrothermal pretreatment hydrolysate, by cell counting in an optical microscope. Absorbance and cell number were converted into cell dry weight (g/L) using a conversion factor. Protease and lipase activities were quantified using azocasein (Sigma) and 4-nitrophenyl butyrate as substrates, respectively, using the methods previously described in Chapter 3.

After 48 h of cultivation in batch cultures with the hydrolysate from hydrothermal pretreated OC, yeast cells were separated from the culture medium by centrifugation and lyophilized. Microbial lipids from lyophilized biomass were extracted with methanol and chloroform (1:1, v/v) and lipid quantification was performed with phospho-vanillin colorimetric assay as described by Lopes et al. (2018). Changes in color after incubation of microbial lipids with phospho-vanillin were measured at 490 nm in a microplate reader. A calibration curve with olive oil as substrate was used to convert absorbance into lipids concentration (g/L). Fatty acid profile of the microbial lipids was determined by quantification of FAMEs as described in Chapter 4. Total nitrogen in the lyophilized yeast biomass was determined by the Kjeldahl method and nitrogen was converted into crude protein using a factor of 6.25.

6.2.9 Statistical analysis

The results are presented as mean ± standard deviation (SD) of two independent experiments. Experimental data were subjected to one-way analysis of variance (ANOVA) and Tukey's test for multiple comparison using the statistical software GraphPad Prism. A confidence level of 95% was selected for the statistical analysis.

6.3 RESULTS AND DISCUSSION

6.3.1 Hydrothermal pretreatment

The by-product from olive mill industries used in this work was characterized by around 26% of polysaccharides (Table 6.1), which have the potential to be converted into fermentable sugars. In comparison to other by-products from agro-industrial activities, OC has a low content of polysaccharides (Melati et al., 2019). However, since this material, which is produced in high quantities in Mediterranean countries, is underexplored and may have a negative environmental impact, the exploitation of several strategies for its valorization is paramount. Besides carbohydrates, OC is also characterized by a high content of lignin, which is in accordance with other reports (Leite et al., 2016; Martínez-Avila et al., 2021). The presence of lignin contributes to the recalcitrance of lignocellulosic biomass, impairing the access of enzymes or solvents to the cellulosic fraction. Pretreatments are used to overcome this issue, resulting in structural changes and higher sugar yields after enzymatic hydrolysis (Melati et al., 2019).

In the present Chapter, hydrothermal pretreatment was performed in OC and two conditions were tested: non-isothermal pretreatment with a maximum temperature of 190 °C and an isothermal treatment, where maximum temperature (210 °C) was kept for 5 min before cooling down. A slight increase in glucan concentration was detected after the pretreatment at lower temperature (Table 6.1). Nonetheless, increasing the pretreatment severity resulted in higher glucan concentration, reaching a 48% increase compared to the raw OC. Although xylan concentration in raw OC and in the remaining solids after the pretreatment at 190 °C was similar (Table 6.1), 46% of this polysaccharide was solubilized during this hydrothermal pretreatment. Moreover, the higher temperature resulted in the hydrolysis of 86% of the xylan present in raw OC and arabinan and acetyl groups were not detected in these conditions. Manzanares et al. (2020) also observed an increase in glucan concentration and, on the other hand, a solubilization of hemicellulose in OC with the increase in pretreatment severity. Moreover, increasing the severity of the pretreatment also resulted in higher lignin percentage in the pretreated solid compared to the raw OC, a result that has been reported by other authors during the hydrothermal pretreatment of OC (Manzanares et al., 2020; Miranda et al., 2019). However, taking into account the content of lignin in raw OC and in the remaining solids, around 25% of lignin was solubilized during both pretreatments. The higher lignin percentage observed in the remaining solids could be associated with the solubilization of polysaccharides and/or the formation of pseudo-lignin during the hydrothermal pretreatment that usually deposit on the cell surface (Pu et al., 2013). While some authors reported that pseudo-lignin formation can have a negative impact in enzymatic hydrolysis (Hu et al., 2012), others argued that the formation

of these structures and their relocation can alter the cell wall matrix structure, improving enzymes accessibility to cellulose without lignin removal (Donohoe et al., 2008).

Table 6.1: Chemical composition (% dry weight) of OC with and without hydrothermal pre-treatment, solid recovery yields (SRY) and severity factor.

Components (% dry weight)	**OC**	**Hydrothermal pretreatment**	
		190 °C	**210 °C**
Glucan	7.7 ± 0.2[a]	10 ± 1[b]	14.9 ± 0.5[c]
Xylan	12.9 ± 0.3[a]	13 ± 1[a]	3.7 ± 0.2[b]
Arabinan	5 ± 1[a]	0.7 ± 0.2[b]	ND
Acetyl groups	1.0 ± 0.6[a]	2.4 ± 0.2[b]	ND
Klason lignin	40 ± 2[a]	55 ± 5[b]	62 ± 2[c]
Solid yield	-	54 ± 2[a]	49 ± 3[a]
Severity factor	-	3.3	4.15

N.D.: not detected
Values represent the mean and SD from two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).

In summary, the pretreatment with higher severity factor resulted in increased hemicellulose solubilization, which is demonstrated by the decrease in xylan, arabinan and acetyl groups concentrations in the solids obtained after hydrothermal pretreatment. In fact, Freitas et al. (2022) observed that higher polysaccharide decomposition in OC was attained with severity factors above 4.0. As expected, the solid recovery yield in the isothermal pretreatment was slightly lower than the non-isothermal one, however, these differences were not statistically significant.

6.3.2 ILs pretreatment

In the present work, cholinium glycinate [N1112OH][Gly] was selected for the pretreatment of OC. These solvents are considered bio-ILs due to their lower toxicity compared to other ILs, such as Imidazolium-based ILs (Zhang et al., 2021). Moreover, [N1112OH][Gly] has been successfully used in brewer's spent grain fractioning (Outeiriño et al., 2019a, 2019b). The impact of pretreatment duration and temperature on OC composition after ILs pretreatment was evaluated and the results are present in Table 6.2. Pretreatments performed at low pretreatment times (2 and 4 h) resulted in low lignin

solubilization, between 35 – 48%, and the concentration of this component slightly increased in the pretreated solid compared to the raw material. Moreover, in some conditions tested at low pretreatment time, a reduction in glucan concentration was also detected. Increasing the pretreatment duration resulted in a significant decrease in lignin content in pretreated OC, with 84% of the lignin being solubilized after 16 h at 90 °C compared to the content of lignin in the raw OC (Table 6.1, Table 6.2). Regarding glucan, a concentration of this polysaccharide was observed in the pretreated solids compared to untreated OC and, once again, higher percentage was attained with the longer pretreatment duration, reaching a 1.8-fold increase in glucan concentration after 16 h of pretreatment. Regardless of the duration and temperature, an increase in xylan percentage was detected after ILs pretreatment, ranging from 1.5 to 2-fold increase compared to raw OC. Despite the higher percentage of these polysaccharides in the recovered solids after ILs pretreatment, solubilization of xylan and glucan was also observed in these conditions, however, this occurred in a lower degree. These results can be explained by the ability of cholinium-based ILs to solubilize lignin and their low solubility towards hemicellulose and cellulose (Zhang et al., 2021). Using cholinium amino acids-based ILs, Hou et al. (2012) pretreated rice straw for 24 h at 90 °C and observed a percentage of delignification ranging from 41 to 60% followed by an increase in glucan concentration compared to the untreated material. Moreover, shorter pretreatment of 6 h also resulted in lignin removal between 50 and 63% in sugarcane bagasse pretreated with six cholinium amino acids-based ILs (Hou et al., 2013). These authors also reported that mixture of ILs with water, which can reduce the viscosity and the pretreatment costs compared to pure ILs, also improved saccharification after enzymatic hydrolysis despite the lower delignification in comparison to the delignification obtained with pure ILs.

Table 6.2: Chemical composition (% dry weight) of OC after pretreatment with [N1112OH][Gly].

Components (%	**90 °C**				**110 °C**	
dry weight)	**2h**	**4h**	**8h**	**16h**	**2h**	**4h**
Glucan	7.6 ± 0.2	3.33 ± 0.01	10.5 ± 0.2	13.6 ± 0.3	6.7 ± 0.1	9.04 ± 0.03
Xylan	19.76 ± 0.04	24.9 ± 0.3	23.9 ± 0.1	25.8 ± 0.3	21.1 ± 0.1	24.4 ± 0.1
Arabinan	4.62 ± 0.01	6.6 ± 0.1	5 ± 0	4.81 ± 0.01	4.82 ± 0.01	4.04 ± 0.01
Acetyl groups	ND	4.07 ± 0.02	2.04 ± 0.01	1.014 ± 0.002	1.21 ± 0.01	1 ± 0
Klason lignin	43 ± 3	48 ± 3	26.4 ± 0.1	17 ± 1	46 ± 1	43.01 ± 0.02
Solid recovery	51 ± 6	54 ± 19	40 ± 2	38 ± 1	45 ± 7	51 ± 7

ND: not detected. Values represent the mean and SD from two independent experiments.

Overall, pretreatment with higher temperatures did not improve lignin solubilization and a slight increase in lignin content in the remaining solid was observed in the experiments performed at 110 °C. Since the temperature can have a great impact in the pretreatment outcome, the effect of this parameter was studied, using temperatures between 60 and 150 °C, in the pretreatment of brewer's spent grain with [N1112OH][Gly] (Outeiriño et al., 2019a). The authors reported that higher percentage of Klason lignin removal (72%) was achieved at 90 °C. While the lower pretreatment duration at 90 °C had similar results to the ones obtained for 110 °C concerning lignin content, increasing the duration to 8 and 16 h led to significant lignin solubilization and an increased percentage of glucan. Moreover, increasing the duration of pretreatment led to a lower solid recovery percentage (Table 6.2).

The two pretreatments used in this work to increase enzymes' accessibility to cellulose of OC during enzymatic hydrolysis impacted the chemical composition of the remaining solids differently. While hydrothermal pretreatment (210 °C/5 min) led to high xylan solubilization, resulting in a solid with higher percentage in lignin and glucan (Table 6.1), pretreatment of OC with ILs significantly reduced the lignin content of the solid and improved polysaccharides' concentration (Table 6.2). Likewise, increased glucan concentration was attained with hydrothermal pretreatment and, on the other hand, ILs pretreatment was more effective in delignification. The use of lower temperatures in ILs constitutes an advantage compared to the hydrothermal pretreatment, which required higher temperatures to affect the composition of OC. Despite this fact, ILs require longer pretreatment duration, which increases energy demands and, together with the high price of ILs, can increase the overall costs of the pretreatment.

6.3.3 Enzymatic hydrolysis

After selection of the ILs and hydrothermal pretreatment conditions that resulted in higher glucan content in OC, enzymatic hydrolysis was performed to release glucose and xylose from the polysaccharides that remained in this material. Untreated OC has also hydrolyzed in the same conditions as a control and resulted in a hydrolysate with 5 g/L of glucose (Table 6.3). Considering the initial concentration of glucan in the untreated material, a glucose conversion of 39% was observed. Xylose, acetic and formic acids were also detected in the hydrolysate but at lower concentrations compared to the pretreated OC. Moreover, pretreatment of the olive by-product improved sugar release compared to untreated OC, resulting in 3.3 and 4-fold increase in xylose content after hydrothermal and ILs pretreatments, respectively. While formic acid content was similar in all the experiments, acetic acid concentration was higher in the hydrolysate from OC after hydrothermal pretreatment. Taking into account

the glucan content of these pretreated materials, OC processing resulted in a saccharification of 47% and 49% after hydrothermal and ILs pretreatments, respectively.

Table 6.3: Chemical composition (g/L) of the hydrolysate obtained after enzymatic hydrolysis of pre-treated OC. Untreated OC was used as a control.

Components (g/L)	**Untreated OC**	**[N1112OH][Gly]**	**Hydrothermal pretreatment**
Glucose	5.1 ± 0.1[a]	8.0 ± 0.1[b]	9 ± 2[b]
Xylose	1.0 ± 0.2[a]	3.8 ± 0.2[b]	4 ± 1[b]
Acetic Acid	0.3 ± 0.1[a]	0.3 ± 0.2[a]	1.4 ± 0.4[b]
Formic acid	0.3 ± 0.0[a]	0.1 ± 0.0[a]	0.3 ± 0.1[a]
5-HMF	ND	ND	0.16 ± 0.03
Furfural	ND	ND	0.15 ± 0.02

ND: not detected

Values represent the mean and SD from two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).

Although, to our knowledge, there are no reports on OC pretreatment with ILs, this method improved glucan saccharification of brewer's spent grain, which had previously been a substrate in SSF, from 14% without ILs pretreatment to 38% after pretreatment with cholinium glycinate (Outeiriño et al., 2019b). Moreover, in another work from the same authors, a greatly improvement in glucan saccharification of brewer's spent grain was achieved after ILs pretreatment, reaching 94% of glucan conversion into glucose (Outeiriño et al., 2019a). Pretreatment of sugarcane bagasse (Hou et al., 2013) and rice straw (Hou et al., 2012) with cholinium-based ILs also improved the materials' saccharification, resulting in glucose yields of around 80%. In these studies, enzymatic hydrolysis was performed with low solid loadings and, in some cases, with high enzyme concentration, which could substantially improve substrate saccharification. Thus, optimization of ILs pretreated-OC enzymatic hydrolysis should be performed to enhance sugars recovery. Conversely to ILs pretreatment, some studies employed hydrothermal pretreatments for the fractioning of olive mill by-products. Cuevas et al. (2015) reported a glucose conversion of 22.3% after enzymatic hydrolysis with 10% (w/v) of olive stones submitted to hydrothermal pretreatment with a severity factor of 4.07. Increasing the severity factor to 4.81 resulted in higher glucose conversion (54.3%). Moreover, enzymatic hydrolysis of extracted OC pretreated for 130

min at 130 °C led to a 40% glucose conversion at a solid loading of 20% (w/v) (Miranda et al., 2019). However, the authors reported conversions of 80 – 90% at high solid loadings by testing different enzymatic cocktails, showing that the enzymes' selected can have a significant influence in enzymatic hydrolysis yields. While enzymatic hydrolysis at high solid loadings can result in hydrolysates with high sugars concentrations and reduce the water employed in the process, problems related to mass transfer and viscosity can impair this process, affecting sugar yields (Modenbach and Nokes, 2013). Enzymatic hydrolysis with 5% (w/v) of OC after hydrothermal pretreatment led to a glucose conversion between 35.9 and 94% depending on the pretreatment severity factor (Manzanares et al., 2020). Moreover, the same authors performed an extraction with water before the pretreatment and this step alone improved enzymatic hydrolysis yield in 1.6-fold. Similarly, a water extraction step was also performed before phosphoric acid and hydrothermal pretreatments of olive tree pruning (Romero-García et al., 2022). This step allowed the removal of extractives that, during pretreatment, can lead to the formation of pseudo-lignin, which can impair enzyme access to cellulose resulting in lower conversion rate (Ballesteros et al., 2011). In the present work, OC was not submitted to any processing before pretreatment, which could have some negative effect on enzymatic hydrolysis and sugar yields. However, water extraction of OC with a solid loading of 10% (w/w) was performed to characterize the soluble compounds of this material and the extract obtained had 9 g/L and 1.2 g/L of glucose and xylose, respectively. Thus, an extraction step of OC could further improve the fractioning of this by-product, resulting in a liquid extract with promising applications in SmF with yeasts or other microorganisms.

Enzymatic hydrolysis is the last stage of lignocellulosic biomass processing into monomeric sugars that can be used as carbon source in several bioprocesses for value-added compounds production. In the present study, 10% (w/w) pretreated OC was submitted to enzymatic hydrolysis, resulting in the production of hydrolysates with 8 and 9 g/L of glucose for the hydrothermal and ILs pretreated OC, respectively. While both pretreatments employed in this study improved cellulose conversion in comparison to untreated OC, several aspects could be considered to further increase OC saccharification, including optimization of solid and enzyme loadings, test different enzymatic cocktails and assess the influence of water extraction before OC pretreatment.

6.3.4 OC hydrolysates as culture medium for *Y. lipolytica* W29

After enzymatic hydrolysis of OC submitted to hydrothermal or ILs pretreatment, the potential of the hydrolysates as carbon source for *Y. lipolytica* W29 growth was examined. The first experiments were performed with the hydrolysate obtained from ILs pretreated OC in 96-wells microplates to assess the influence of medium supplementation on yeast growth after 33 h of incubation (Figure 6.1A). Ammonium sulfate, YNB and CSL were the supplements selected. The latter is a by-product from corn processing with high content in free amino acids, vitamins and lactic acid and the use of this material as supplement for microbial growth allows, in one hand, the valorization of agro-industrial by-products, and on the other hand, a reduction in bioprocesses production costs. Experiments without medium supplementation were performed as a control and a culture medium with 8 g/L glucose was used as a standard assay. Overall, yeast biomass was significantly higher ($p < 0.05$) when the OC hydrolysate was used as culture medium compared to the experiments with glucose as carbon source (Figure 6.1A). Highest yeast biomass was registered with OC hydrolysate supplemented with 2 g/L CSL and 5 g/L ammonium sulphate and the OD values obtained in this condition corresponded to 1.6 g/L of yeast biomass. Moreover, in the cultures medium without medium supplementation, OD was 8.7-fold higher in the experiment with OC hydrolysate in comparison to yeasts cultivated in the glucose medium. This outcome can be explained by the presence of other compounds, excluding glucose, in the hydrolysate, for instance, xylose, organic acids and phenolic compounds, which could contribute to the higher yeast growth observed in these conditions.

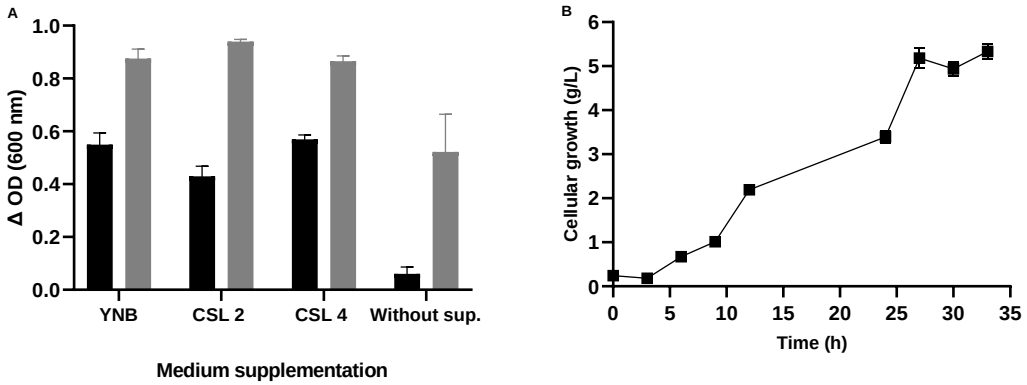

Figure 6.1: Cellular growth of *Y. lipolytica* W29 in batch cultures in 96-well microplates (A) and in Erlenmeyer flasks (B) for 33h. Microplate assays were performed with glucose (black bars) or OC hydrolysate obtained after treatment with ILs (grey bars). Culture media were supplemented with 6.7 g/L YNB (YNB), 2 g/L CSL and 5 g/L ammonium sulphate (CSL 2) or 4 g/L CSL and 5 g/L ammonium

sulphate (CSL 4). Culture media without supplementation was used as a control. Batch cultures were supplemented with 2 g/L CSL and 5 g/L ammonium sulphate. Experiments in microplates were performed in triplicate.

Despite this result, the OD measured in OC hydrolysate without supplements was around 38% lower than the results obtained with medium supplementation, showing that this step is fundamental for high biomass production. Moreover, the utilization of YNB and CSL at higher concentrations (4 g/L) did not improve cellular growth in the conditions tested in the present work. For this reason, batch cultures with OC hydrolysate were performed with 2 g/L CSL and 5 g/L ammonium sulphate for 33 h at increased volume in Erlenmeyer's flasks (Figure 6.1B). After a lag phase in the first 3 h of cultivation, yeast biomass concentration increased until 27 h and it appears that, at this point, cells entered the stationary phase. By the end of the fermentation process, 5.3 g/L of yeast biomass was attained, which is 3.3-fold higher than the results obtained with the same medium supplementation in the microplate experiments. In microplate assays, oxygen transfer and medium agitation is limited, which can have a negative effect on cellular growth. However, these small-scale assays allow the utilization of a reduced volume of culture medium, which is an advantage in this study since the pretreatment with ILs was performed with a small sample of OC. Moreover, several conditions were tested at the same time, facilitating the optimization of OC hydrolysate supplementation.

By the end of cultivation, glucose and xylose were not detected in the culture medium, showing that *Y. lipolytica* W29 was able to metabolize these compounds present in OC hydrolysate. *Yarrowia lipolytica* is known for the metabolization of hexose sugars such as glucose, however, assimilation of xylose, a pentose sugar, is less efficient. In yeasts, xylose is converted into D-xylitol by xylose reductase after entering the cells and xylitol dehydrogenase converts D-xylitol into D-xylulose. Lastly, before entering the pentose phosphate pathway, D-xylulose is converted into D-xylulose-5-phosphate by xylulokinase (Ryu and Trinh, 2018). Moreover, it appears that the rate limiting step that leads to low metabolization of xylose by *Y. lipolytica* is related to xylose transportation into the cell and the expression of xylitol dehydrogenase (Ryu and Trinh, 2018). In fact, some studies reported that this yeast is unable to grow in a medium with xylose as the only carbon source (Ledesma-Amaro et al., 2016; Zhao et al., 2015). In the present study, glucose was the main sugar present in the OC hydrolysate (Table 6.3) and *Y. lipolytica* W29 was able to metabolize both glucose and xylose.

Besides the hydrolysate obtained after enzymatic hydrolysis of ILs pretreated OC, the hydrolysate that resulted from hydrolysis of OC after hydrothermal pretreatment was also used as culture medium in

batch cultures with *Y. lipolytica* W29 (Figure 6.2). In these experiments, OC hydrolysate based medium was composed of the hydrolysate supplemented with 2 g/L CSL and 5 g/L ammonium sulphate. Additional experiments were performed adding olive oil, a well-known lipase inducer (Lopes et al., 2008), to the medium, followed by another experiment with further addition of arabic gum, an emulsifying agent. In the first 24 h of cultivation, cell growth was very similar regardless of the supplementation used (Figure 6.2A). From this point until the end of the fermentation, a slightly higher growth was observed with OC hydrolysate with olive oil but these differences were not statistically significant ($p < 0.05$). Furthermore, final yeast biomass concentration obtained in these experiments was between 8.6 g/L and 10 g/L. Glucose and xylose concentration was monitored through time and the results are represented in Figure 6.2B. Regardless of the medium supplementation used, a decline in glucose concentration was detected in the first 24 h and, after this point, glucose depletion was observed. In contrast, xylose concentration remained stable in the beginning of the experiments and, after glucose consumption, metabolization of this pentose occurred. By the end of the fermentation process, 75% of the xylose present in the OC hydrolysate was metabolized by *Y. lipolytica* W29. Similarly, in reports on metabolic engineering of *Y. lipolytica* to efficiently metabolize xylose, Ledesma-Amaro et al. (2016) observed that the wild strain was able to metabolize xylose after glucose depletion.

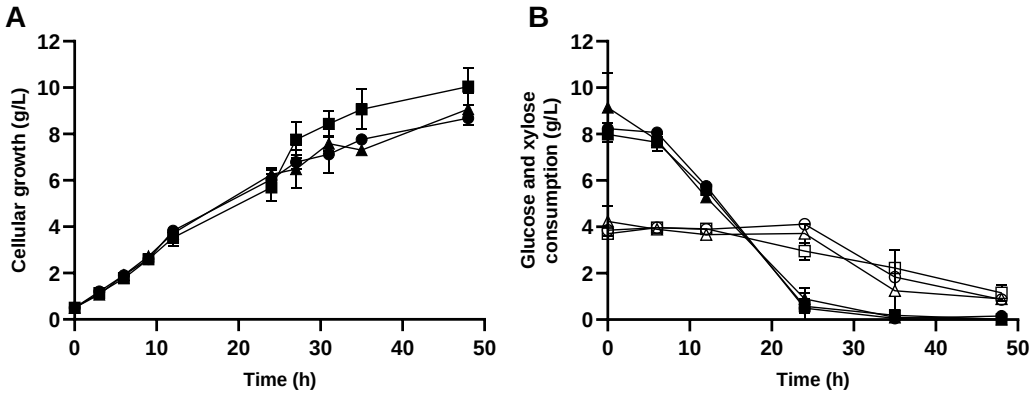

Figure 6.2: Time course of cellular growth (A), glucose (filled symbols) and xylose (empty symbols) consumption (B) by *Y. lipolytica* W29 in batch cultures with OC hydrolysate based medium (●,○), with 7 g/L olive oil (■,□) and with 7 g/L olive oil plus 5 g/L arabic gum (▲,△). Experiments were performed in duplicate. The error bars represent the SD of two independent experiments.

Lopes et al. (2008) and Braga et al. (2012) used a defined culture medium supplemented with olive oil and arabic gum to induce lipase production by *Y. lipolytica* W29. Despite the medium supplementation with olive oil and arabic gum in the present work, lipase activity in the extracellular medium was not detected until the end of cultivation. Thus, the presence of glucose in OC hydrolysate could have a negative impact on this enzyme production. It appears that hexokinase is involved in glucose catabolite repression of *LIP2,* the gene coding for the extracellular lipase most secreted by *Y. lipolytica*, in media containing glucose (Fickers et al., 2005). This repression could result in the metabolization of glucose by yeast cells and, only after its depletion, consumption of olive oil would begin, resulting in lipase induction. Furthermore, Najjar et al. (2011) compared lipase production by *Y. lipolytica* CBS 7504 in different medium containing glucose, olive oil or a combination of both as carbon sources. The authors observed that in the culture medium containing olive oil and glucose, lipase activity was kept at low levels during the experiment and a delayed peak was registered at 70 h, being 85% lower in comparison to the lipase activity peak achieved in medium containing only olive oil (reached after 28 h). Moreover, in these experiments, total lipase activity was measured while, in the present study, lipase activity was only quantified in the extracellular medium. In the beginning of cultivation, lipase in mainly cell-bounded, being released into the culture medium when the concentration of the carbon source is decreased (Najjar et al., 2011; Pereira-Meirelles et al., 2000). Additionally, the release of these lipolytic enzymes can occurred in stationary or late stationary phase (Pereira-Meirelles et al., 2000). Likewise, in the present study, it is possible that, while no lipase was detected in the extracellular medium, this enzyme could be bounded to the yeast cell and would only be released into the extracellular medium latter in the fermentation process when the cells entered the stationary phase. In these batch cultures performed in this study, it appears that *Y. lipolytica* cells had not reached stationary phase after 48 h of cultivation (Figure 6.2A). Thus, increase in cultivation time and quantification of total lipase production should be done to further study lipase production by *Y. lipolytica* W29 using hydrolysates from olive mill by-products. Moreover, some studies reported the utilization of Tween 20 (De Almeida et al., 2013) and Tween 80 (Dalmau et al., 2000) for lipase induction by yeasts, thus, optimization of the surfactant used in the culture medium could also be performed.

After 48 h of cultivation in medium with olive oil, appearance of lipidic bodies inside *Y. lipolytica* W29 cells was detected (data not shown). Lipid accumulation by *Y. lipolytica* cells can occur by two different pathways. While *de novo* synthesis requires the production of fatty acids precursors, such as acetyl-CoA, the *ex novo* route involves the incorporation of fatty acids from the growth medium and this pathway is triggered when oleaginous microorganisms grow in a medium rich in oils (Beopoulos et al.,

2009; Gonçalves et al., 2014). In the present work, in the cells cultivated in OC hydrolysate based medium, intracellular lipids corresponded to 5% (w/w) of the dry yeast biomass. The culture medium supplementation with nitrogen sources in these experiments resulted in a low C/N ratio, which are unfavored conditions for lipid biosynthesis and accumulation via *de novo* route since it occurs during nitrogen-limiting conditions (Beopoulos et al., 2009). Conversely, when *Y. lipolytica* was cultivated in the presence of olive oil, a 4.4-fold increase in the content of microbial lipids was observed, reaching 22% (w/w) in both experiments. This result demonstrated that *Y. lipolytica* was able to assimilate lipids from olive oil in the culture medium. Similarly, growth in culture medium with olive oil as carbon source resulted in 20.7% and 24.4% (w/w) of microbial lipids using *Y. lipolytica* KKP 379 (Fabiszewska et al., 2019) and *Y. lipolytica* ACA-DC 50109 (Bellou et al., 2016), respectively. Moreover, the presence of OMWW in the growth medium of *Y. lipolytica* W29 resulted in intracellular lipid accumulation ranging from 9 to 34 % (w/w) (Sarris et al., 2011). Fatty acids profile of the *Y. lipolytica* W29 biomass was analyzed and compared to the profile of olive oil and the results are represented in Table 6.4. The olive oil used in these experiments is characterized by a high percentage of oleic acid (C18:1), followed by palmitic (C16:0), steric (C18:0) and linoleic (C18:2) acids. Cultivation of *Y. lipolytica* W29 in OC hydrolysate supplemented with olive oil resulted not only in fatty acids incorporation by yeast cells but also in their biomodification in comparison to the initial substrate. *Yarrowia lipolytica* cells were characterized by a lower percentage of saturated fatty acids compared to olive oil. In particular, a reduction of around 50% in steric acid was observed and, regarding palmitic acid, a 50% and 32% reduction was detected compared to olive oil when yeast cells were cultivated with olive oil and with olive oil plus arabic gum, respectively. Moreover, cultivation of *Y. lipolytica* W29 in OC hydrolysates supplemented with olive oil resulted in a dry yeast biomass with higher percentage of the unsaturated fatty acids oleic and linoleic, accounting, on average, for 89% of the total fatty acids. Biomass from *Y. lipolytica* KKP 379 grown in olive oil containing medium was also characterized by a higher percentage of oleic acid and a 66% lower content in palmitic acid compared to the initial substrate (Fabiszewska et al., 2019).

Table 6.4: Long chain fatty acids (LCFAs) profile of olive oil and yeast biomass cultivated in a culture medium supplemented with olive oil or olive oil plus arabic gum.

LCFAs (%)	Olive oil	Supplementation with olive oil	Supplementation with olive oil and arabic gum
C16:0	12.0 ± 0.1[a]	6 ± 1[b]	8.2 ± 0.4[c]
C16:1	0.88 ± 0.01[a]	0.9 ± 0.1[a]	0.92 ± 0.05[a]
C18:0	6 ± 1[a]	2.7 ± 0.4[b]	3 ± 1[b]
C18:1	75 ± 2[a]	82 ± 1[b]	79 ± 1[b]
C18:2	6.0 ± 0.1[a]	8 ± 1[b]	8.7 ± 1[b]

Values represent the mean and SD from two independent experiments. Values with the same letter within the same row are not statistically different ($p > 0.05$).

Microbial biomass is often characterized by a high content of protein and is referred as single cell protein. Medium without olive oil supplementation resulted in yeast biomass with the highest content in protein, reaching 35% (w/w). In contrast, *Y. lipolytica* cells grown in the presence of olive oil and olive oil plus arabic gum presented a slight lower protein content of 26 % and 28 % (w/w), respectively. Growth of *Y. lipolytica* for biomass production has been performed using several by-products resultant from industrial and agro-industrial activities. Growth of *Y. lipolytica* in food wastes resulted in yeast biomass with a crude protein content ranging from 20.04% to 24.17% (w/w) (Yang et al., 2022). In the same study the authors reported that anaerobic digestion of the food wastes before SmF with the oleaginous yeast improved cell growth and the protein content of the biomass, reaching 39% (w/w) after 108 h of fermentation. Moreover, biomass of *Y. lipolytica* A101 cultivated in agro-industrial wastes hydrolysates was characterized by a protein content between 30.5 and 45 % (w/w) (Drzymała et al., 2020). The longer cultivation time (120 h) could explain the higher protein content in these experiments compared to the protein content obtained in the present work.

Yarrowia lipolytica is a microorganism with GRAS status, being considered safe for food and feed (Groenewald et al., 2014). Although extracellular lipase activity was not detected, which was the main goal of these experiments, the yeast biomass obtained at the end of SmF was characterized by a high content in crude protein and, when medium was supplemented with olive oil, with high percentage of lipids. Moreover, the higher accumulation of unsaturated fatty acids in comparison to saturated ones, resulted in the biomodification of the initial substrate and a biomass with improved nutritional properties.

In order to summarize, the work here reported showed that OC can be treated by ILs and hydrothermal processes, but, in the conditions tested, the last pretreatment led to the highest content in glucans of the treated OC (Figure 6.3). In contrast, the OC obtained after ILs pretreatment presented the lowest content of lignin. From the enzymatic hydrolysis of those polysaccharides resulted OC hydrolysates and higher glucose was attained from hydrothermal pretreated OC. Moreover, *Y. lipolytica* W29 was able to grow in these hydrolysates without any further detoxication steps. Biomass yield (cell mass per glucose and xylose consumed) in the hydrolysate of ILs pretreated OC reached 0.45 g/g after 33 h and a 69% increase in this parameter was observed in experiments with the hydrolysate from hydrothermal pretreatment (supplemented with CSL and ammonium sulphate) at around the same time point. This result could reveal that sugar conversion into yeast biomass was more efficient in the latter hydrolysate or, on the other hand, that *Y. lipolytica* W29 was able to metabolize other biocompounds present in this hydrolysate. In fact, Dias et al. (2023) observed furfural, 5-HMF and acetic and formic acids assimilation by *Y. lipolytica* W29 cultivated in a culture medium mimicking a hydrolysate obtained from lignocellulosic biomass pretreatment. As depicted in Table 6.3, the hydrolysate obtained from hydrothermal pretreated OC presents higher concentrations of acetic and formic acids and residual values of 5-HMF and furfural were also detected conversely to the ILs hydrolysate. After 48 h of cultivation, 5-HMF and furfural were metabolized by the yeast and, although quantification of acetic acid and formic acid was unfeasible due to the presence of other compounds in the culture medium, it is possible that *Y. lipolytica* W29 metabolized these compounds resulting in higher biomass concentrations.

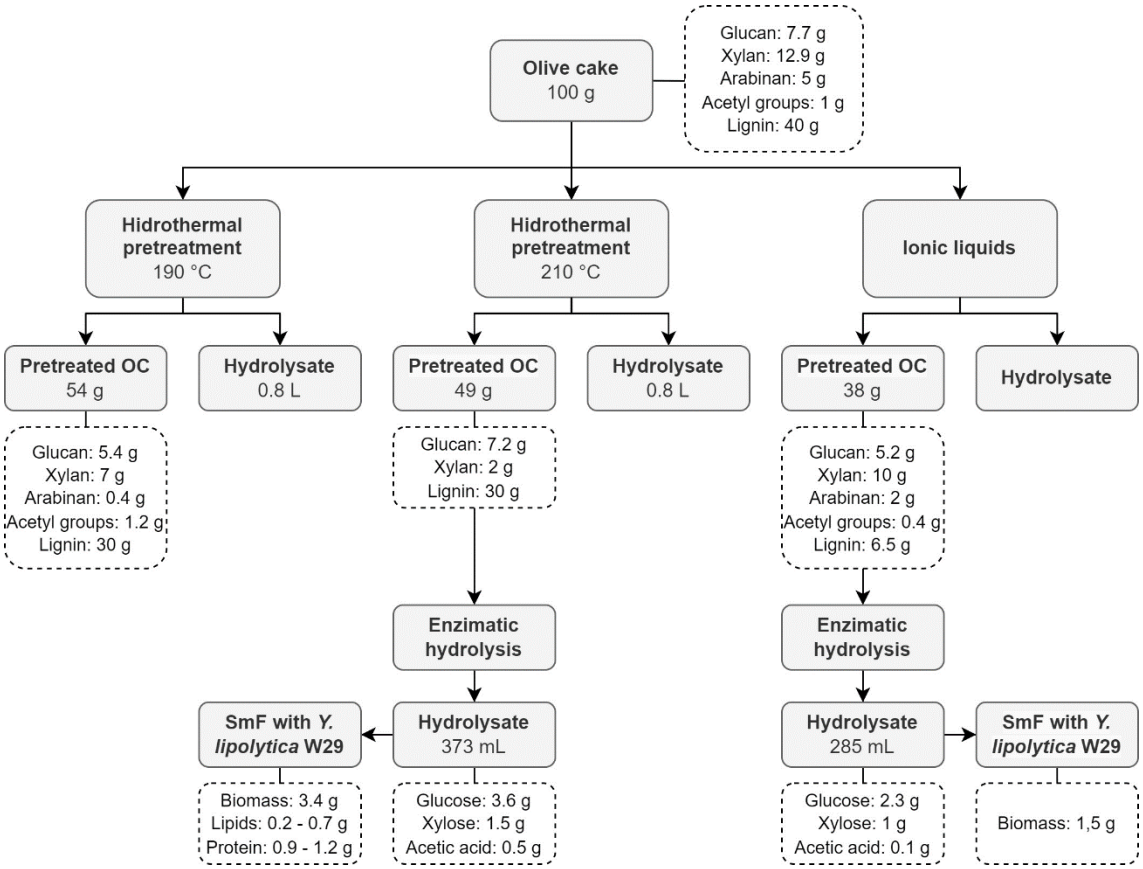

Figure 6.3: Mass balance of 100 g of OC treated with ILs and hydrothermal pretreatments, followed by enzymatic hydrolysis and SmF with OC hydrolysate based medium by *Y. lipolytica* W29.

6.4 CONCLUSIONS

Pretreatment of OC before enzymatic hydrolysis was successfully performed using ILs and hydrothermal pretreatments. Pretreatment with ILs led to a significant delignification of OC, showing that this is a promising strategy for OC fractioning, since this by-product has a high content in lignin. Moreover, hydrothermal pretreatment at 210 °C resulted in higher glucan percentage in the pretreated OC compared to raw OC. Although the chemical composition of pretreated OC differed depending on the pretreatment employed, both methods improved sugar release during enzymatic hydrolysis compared to the raw OC. *Yarrowia lipolytica* W29 was able to grow in the OC hydrolysates regardless of the pretreatment used and the main sugars, glucose and xylose, were metabolized by the yeast. Yeast biomass obtained after 48 h of cultivation was rich in crude protein and, in the presence of olive oil, high content in lipids and improvement of unsaturated fatty acids profile was observed.

6.5 REFERENCES

AOAC, 18th editi. ed, 2005. , Official methods of analysis of the Association of Official Analytical Chemists. Washington DC, USA.

Ballesteros, I., Ballesteros, M., Cara, C., Sáez, F., Castro, E., Manzanares, P., Negro, M.J., Oliva, J.M., 2011. Effect of water extraction on sugars recovery from steam exploded olive tree pruning. Bioresour. Technol. 102, 6611–6616. https://doi.org/10.1016/j.biortech.2011.03.077

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., Chardot, T., Nicaud, J.M., 2009. *Yarrowia lipolytica*: A model and a tool to understand the mechanisms implicated in lipid accumulation. Biochimie 91, 692–696. https://doi.org/10.1016/j.biochi.2009.02.004

Braga, A., Gomes, N., Belo, I., 2012. Lipase induction in *Yarrowia lipolytica* for castor oil hydrolysis and its effect on γ-decalactone production. J. Am. Oil Chem. Soc. 89, 1041–1047. https://doi.org/10.1007/s11746-011-1987-5

Costa, A.R., Salgado, J.M., Lopes, M., Belo, I., 2022. Valorization of by-products from vegetable oil industries: Enzymes production by *Yarrowia lipolytica* through solid state fermentation. Front. Sustain. Food Syst. 6. https://doi.org/10.3389/fsufs.2022.1006467

Cuevas, M., García, J.F., Hodaifa, G., Sánchez, S., 2015. Oligosaccharides and sugars production from olive stones by autohydrolysis and enzymatic hydrolysis. Ind. Crops Prod. 70, 100–106. https://doi.org/10.1016/j.indcrop.2015.03.011

Dalmau, E., Montesinos, J.L., Lotti, M., Casas, C., 2000. Effect of different carbon sources on lipase production by *Candida rugosa*. Enzyme Microb. Technol. 26, 657–663. https://doi.org/10.1016/S0141-0229(00)00156-3

De Almeida, A.F., Taulk-Tornisielo, S.M., Carmona, E.C., 2013. Influence of carbon and nitrogen sources on lipase production by a newly isolated *Candida viswanathii* strain. Ann. Microbiol. 63, 1225–1234. https://doi.org/10.1007/s13213-012-0580-y

Deive, F.J., Ruivo, D., Rodrigues, J. V., Gomes, C.M., Sanromán, M.Á., Rebelo, L.P.N., Esperança, J.M.S.S., Rodríguez, A., 2015. On the hunt for truly biocompatible ionic liquids for lipase-catalyzed reactions. RSC Adv. 5, 3386–3389. https://doi.org/10.1039/c4ra15021j

Dias, B., Fernandes, H., Lopes, M., Belo, I., 2023. *Yarrowia lipolytica* produces lipid-rich biomass in medium mimicking lignocellulosic biomass hydrolysate. Appl. Microbiol. Biotechnol. 3925–3937. https://doi.org/10.1007/s00253-023-12565-6

Donohoe, B.S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B., 2008. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. Biotechnol. Bioeng. 101, 913–925. https://doi.org/10.1002/bit.21959

Drzymała, K., Mirończuk, A.M., Pietrzak, W., Dobrowolski, A., 2020. Rye and oat agricultural wastes as

substrate candidates for biomass production of the non-conventional yeast *Yarrowia lipolytica*. Sustain. 12, 1–12. https://doi.org/10.3390/su12187704

European Comission, 2023. Olive oil production [WWW Document]. URL https://agridata.ec.europa.eu/extensions/DashboardOliveOil/OliveOilProduction.html (accessed 6.20.23).

Fabiszewska, A., Misiukiewicz-Stępień, P., Paplińska-Goryca, M., Zieniuk, B., Białecka-Florjańczyk, E., 2019. An insight into storage lipid synthesis by *Yarrowia lipolytica* yeast relating to lipid and sugar substrates metabolism. Biomolecules 9. https://doi.org/10.3390/biom9110685

Fickers, P., Nicaud, J.M., Destain, J., Thonart, P., 2005. Involvement of hexokinase Hxk1 in glucose catabolite repression of LIP2 encoding extracellular lipase in the yeast *Yarrowia lipolytica*. Curr. Microbiol. 50, 133–137. https://doi.org/10.1007/s00284-004-4401-9

Filipe, D., Fernandes, H., Castro, C., Peres, H., Oliva-Teles, A., Belo, I., Salgado, J.M., 2020. Improved lignocellulolytic enzyme production and antioxidant extraction using solid-state fermentation of olive pomace mixed with winery waste. Biofuels, Bioprod. Biorefining 14, 78–91. https://doi.org/10.1002/bbb.2073

Freitas, L., Simões, R., Miranda, I., Peres, F., Ferreira-Dias, S., 2022. Optimization of Autohydrolysis of Olive Pomaces to Obtain Bioactive Oligosaccharides: The Effect of Cultivar and Fruit Ripening. Catalysts 12. https://doi.org/10.3390/catal12070788

Gonçalves, F.A.G., Colen, G., Takahashi, J.A., 2014. *Yarrowia lipolytica* and Its Multiple Applications in the Biotechnological Industry. Sci. World J. 1–14. https://doi.org/10.1155/2014/476207

Groenewald, M., Boekhout, T., Neuvéglise, C., Gaillardin, C., Van Dijck, P.W.M., 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

Hayes, R., Warr, G.G., Atkin, R., 2015. Structure and Nanostructure in Ionic Liquids. Chem. Rev. 115, 6357–6426. https://doi.org/10.1021/cr500411q

Hou, X.D., Li, N., Zong, M.H., 2013. Facile and simple pretreatment of sugar cane bagasse without size reduction using renewable ionic liquidswater mixtures. ACS Sustain. Chem. Eng. 1, 519–526. https://doi.org/10.1021/sc300172v

Hou, X.D., Smith, T.J., Li, N., Zong, M.H., 2012. Novel renewable ionic liquids as highly effective solvents for pretreatment of rice straw biomass by selective removal of lignin. Biotechnol. Bioeng. 109, 2484–2493. https://doi.org/10.1002/bit.24522

Hu, F., Jung, S., Ragauskas, A., 2012. Pseudo-lignin formation and its impact on enzymatic hydrolysis. Bioresour. Technol. 117, 7–12. https://doi.org/10.1016/j.biortech.2012.04.037

Kellock, M., Maaheimo, H., Marjamaa, K., Rahikainen, J., Zhang, H., Holopainen-Mantila, U., Ralph, J., Tamminen, T., Felby, C., Kruus, K., 2019. Effect of hydrothermal pretreatment severity on lignin inhibition in enzymatic hydrolysis. Bioresour. Technol. 280, 303–312. https://doi.org/10.1016/j.biortech.2019.02.051

Le Donne, A., Bodo, E., 2021. Cholinium amino acid-based ionic liquids. Biophys. Rev. 13, 147–160. https://doi.org/10.1007/s12551-021-00782-0

Ledesma-Amaro, R., Lazar, Z., Rakicka, M., Guo, Z., Fouchard, F., Coq, A.M.C. Le, Nicaud, J.M., 2016. Metabolic engineering of *Yarrowia lipolytica* to produce chemicals and fuels from xylose. Metab. Eng. 38, 115–124. https://doi.org/10.1016/j.ymben.2016.07.001

Leite, P., Salgado, J.M., Venâncio, A., Domínguez, J.M., Belo, I., 2016. Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. Bioresour. Technol. 214, 737–746. https://doi.org/10.1016/j.biortech.2016.05.028

Lopes, M., Gomes, N., Gonçalves, C., Coelho, M.A.Z., Mota, M., Belo, I., 2008. *Yarrowia lipolytica* lipase production enhanced by increased air pressure. Lett. Appl. Microbiol. 46, 255–260. https://doi.org/10.1111/j.1472-765X.2007.02299.x

Lopes, M., Miranda, S.M., Alves, J.M., Pereira, A.S., Belo, I., 2018. Waste Cooking Oils as Feedstock for Lipase and Lipid-Rich Biomass Production. Eur. J. Lipid Sci. Technol. 1–9. https://doi.org/10.1002/ejlt.201800188

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. Crit. Rev. Biotechnol. 42, 163–183. https://doi.org/10.1080/07388551.2021.1931016

Lopes, V.R.O., Farias, M.A., Belo, I.M.P., Coelho, M.A.Z., 2016. Nitrogen sources on TPOMW valorization through solid state fermentation performed by *Yarrowia lipolytica*. Brazilian J. Chem. Eng. 33, 261–270. https://doi.org/10.1590/0104-6632.20160332s20150146

Manzanares, P., Ballesteros, I., Negro, M.J., González, A., Oliva, J.M., Ballesteros, M., 2020. Processing of extracted olive oil pomace residue by hydrothermal or dilute acid pretreatment and enzymatic hydrolysis in a biorefinery context. Renew. Energy 145, 1235–1245. https://doi.org/10.1016/j.renene.2019.06.120

Martínez-Avila, O., Llimós, J., Ponsá, S., 2021. Integrated solid-state enzymatic hydrolysis and solid-state fermentation for producing sustainable polyhydroxyalkanoates from low-cost agro-industrial residues. Food Bioprod. Process. 126, 334–344. https://doi.org/10.1016/j.fbp.2021.01.015

Melati, R.B., Shimizu, F.L., Oliveira, G., Pagnocca, F.C., de Souza, W., Sant'Anna, C., Brienzo, M., 2019. Key Factors Affecting the Recalcitrance and Conversion Process of Biomass. Bioenergy Res. 12, 1–20. https://doi.org/10.1007/s12155-018-9941-0

Miranda, I., Simões, R., Medeiros, B., Nampoothiri, K.M., Sukumaran, R.K., Rajan, D., Pereira, H., Ferreira-Dias, S., 2019. Valorization of lignocellulosic residues from the olive oil industry by production of lignin, glucose and functional sugars. Bioresour. Technol. 292, 121936. https://doi.org/10.1016/j.biortech.2019.121936

Modenbach, A.A., Nokes, S.E., 2013. Enzymatic hydrolysis of biomass at high-solids loadings - A review. Biomass and Bioenergy 56, 526–544. https://doi.org/10.1016/j.biombioe.2013.05.031

Moftah, O.A.S., Grbavčić, S., Žuža, M., Luković, N., Bezbradica, D., Knežević-Jugović, Z., 2012. Adding value to the oil cake as a waste from oil processing industry: Production of lipase and protease by

Candida utilis in solid state fermentation. Appl. Biochem. Biotechnol. 166, 348–364. https://doi.org/10.1007/s12010-011-9429-2

Moftah, O.A.S., Grbavcic, S.Z., Moftah, W.A.S., Lukovic, N.D., Prodanovic, O.L., Jakovetic, S.M., Kneževic-Jugovic, Z.D., 2013. Lipase production by *Yarrowia lipolytica* using olive oil processing wastes as substrates. J. Serbian Chem. Soc. 78, 781–794. https://doi.org/10.2298/JSC120905005M

Najjar, A., Robert, S., Guérin, C., Violet-Asther, M., Carrière, F., 2011. Quantitative study of lipase secretion, extracellular lipolysis, and lipid storage in the yeast *Yarrowia lipolytica* grown in the presence of olive oil: Analogies with lipolysis in humans. Appl. Microbiol. Biotechnol. 89, 1947–1962. https://doi.org/10.1007/s00253-010-2993-5

Outeiriño, D., Costa-Trigo, I., Paz, A., Deive, F.J., Rodríguez, A., Domínguez, J.M., 2019a. Biorefining brewery spent grain polysaccharides through biotuning of ionic liquids. Carbohydr. Polym. 203, 265–274. https://doi.org/10.1016/j.carbpol.2018.09.042

Outeiriño, D., Costa-Trigo, I., Pinheiro de Souza Oliveira, R., Pérez Guerra, N., Domínguez, J.M., 2019b. A novel approach to the biorefinery of brewery spent grain. Process Biochem. 85, 135–142. https://doi.org/10.1016/j.procbio.2019.06.007

Pereira-Meirelles, F. V., Rocha-Leão, M.H.M., Sant'Anna, G.L., 2000. Lipase location in *Yarrowia lipolytica* cells. Biotechnol. Lett. 22, 71–75. https://doi.org/10.1023/A:1005672731818

Pu, Y., Hu, F., Huang, F., Davison, B.H., Ragauskas, A.J., 2013. Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments. Biotechnol. Biofuels 6, 1–13. https://doi.org/10.1186/1754-6834-6-15

Romero-García, J.M., López-Linares, J.C., Contreras, M. del M., Romero, I., Castro, E., 2022. Exploitation of olive tree pruning biomass through hydrothermal pretreatments. Ind. Crops Prod. 176, 1–9. https://doi.org/10.1016/j.indcrop.2021.114425

Ruiz, H.A., Thomsen, M.H., Trajano, H.L., 2017. Effect of Hydrothermal Pretreatment on Lignin and Antioxidant Activity, Hydrothermal Processing in Biorefineries: Production of Bioethanol and High Added-Value Compounds of Second and Third Generation Biomass. https://doi.org/10.1007/978-3-319-56457-9

Ryu, S., Trinh, C.T., 2018. Understanding functional roles of native pentose-specific transporters for activating dormant pentose metabolism in *Yarrowia lipolytica*. Appl. Environ. Microbiol. 84. https://doi.org/10.1128/AEM.02146-17

Salgado, J.M., Abrunhosa, L., Venâncio, A., Domínguez, J.M., Belo, I., 2014. Screening of winery and olive mill wastes for lignocellulolytic enzyme production from Aspergillus species by solid-state fermentation. Biomass Convers. Biorefinery 4, 201–209. https://doi.org/10.1007/s13399-013-0100-8

Sarris, D., Galiotou-Panayotou, M., Koutinas, A.A., Komaitis, M., Papanikolaou, S., 2011. Citric acid, biomass and cellular lipid production by *Yarrowia lipolytica* strains cultivated on olive mill wastewater-based media. J. Chem. Technol. Biotechnol. 86, 1439–1448. https://doi.org/10.1002/jctb.2658

Usmani, Z., Sharma, M., Gupta, P., Karpichev, Y., Gathergood, N., Bhat, R., Gupta, V.K., 2020. Ionic liquid based pretreatment of lignocellulosic biomass for enhanced bioconversion. Bioresour. Technol. 304, 123003. https://doi.org/10.1016/j.biortech.2020.123003

Wagle, Aditi, Angove, M.J., Mahara, A., Wagle, Amrita, Mainali, B., Martins, M., Goldbeck, R., Raj Paudel, S., 2022. Multi-stage pre-treatment of lignocellulosic biomass for multi-product biorefinery: A review. Sustain. Energy Technol. Assessments 49, 101702. https://doi.org/10.1016/j.seta.2021.101702

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

Zhang, J., Zhang, X., Yang, M., Singh, S., Cheng, G., 2021. Transforming lignocellulosic biomass into biofuels enabled by ionic liquid pretreatment. Bioresour. Technol. 322, 124522. https://doi.org/10.1016/j.biortech.2020.124522

Zhao, C., Gu, D., Nambou, K., Wei, L., Chen, J., Imanaka, T., Hua, Q., 2015. Metabolome analysis and pathway abundance profiling of *Yarrowia lipolytica* cultivated on different carbon sources. J. Biotechnol. 206, 42–51. https://doi.org/10.1016/j.jbiotec.2015.04.005

7 CONCLUSIONS AND FUTURE PERSPECTIVES

In this Chapter are displayed the main conclusions of this work.

Based on the results of obtained, future perspectives and suggestions for future work are also discussed.

7.1 CONCLUSIONS

In the present work, oil cakes from sunflower, rapeseed and olive oil extraction processes proved to be suitable substrates in SSF with *Y. lipolytica* for the production of lipases and proteases without the requirement for any medium supplementation. Moreover, high enzyme activity was achieved in a short period of time and modulation of enzyme production can be attained with different combination of these by-products, resulting in target biocompound production.

Since this oleaginous yeast is unable to produce enzymes that degrade the hemicellulosic fraction of oil cakes, increase in sugar content in the optimum substrate mixture for lipase production was successfully performed with enzymatic hydrolysis using an enzymatic cocktail produced by *A. niger.* While a delay on lipase production was observed, possibly due to the high sugar concentration, a significant increase in the growth of *Y. lipolytica* was detected due to the increased availability of carbon sources in the substrate mixture. Moreover, microwave irradiation of the mixture of OC and SC improved microbial growth and lipase production, showing that the pretreatment could possibly improve the access of the yeast to the substrate mixture and increase the solubility of some biocompounds.

Scale-up of SSF with the optimum mixture for lipase production without substrate pretreatment or supplementation was performed using different substrate loadings and bioreactor designs. With tray bioreactors, it was observed that highest lipase activity was attained with 50 g of dry substrate mixture and oxygen availability plays an important role in the production of this enzyme. Conversely, in horizontal drum bioreactors, a significant increase in *Y. lipolytica* growth was detected, which could be related to the substrate mixing.

To further exploit OC, ILs and hydrothermal pretreatments were selected to reduce the recalcitrant nature of this by-product and increase enzymes' access to cellulose during enzymatic hydrolysis. Both methods successfully altered the chemical composition of OC: hydrothermal pretreatment resulted in a solid with higher glucan content while ILs were more effective in lignin removal. Furthermore, both pretreatments improved sugar release during enzymatic hydrolysis compared to the untreated OC, reaching saccharification percentages close to 50%. *Yarrowia lipolytica* W29 was able to grow in OC hydrolysate based medium and metabolize the biocompounds present it these culture media. In the presence of olive oil, fatty acids from this carbon source were incorporated by the oleaginous yeast resulting in a yeast biomass with higher percentage of unsaturated fatty acids than olive oil. Moreover, yeast biomass obtained in these conditions was also characterized by a high content in protein, showing its promising application in food industries since *Y. lipolytica* is considered safe for human ingestion.

In this work, an attempt for lipase production by *Y. lipolytica* was performed through three different bioprocesses: SSF and semi-solid fermentation with a 50 % (w/w) mixture of OC and SC and SmF with an OC hydrolysate based medium. Highest lipase activity was registered in small scale SSF after two days of incubation and an aqueous extract with 12785 U/L could be obtained in these conditions. Moreover, although a significant reduction in lipase activity was observed in semi-solid fermentation, this process resulted in the highest cellular concentration observed in this work. Thus, high lipase and yeast biomass production could be achieved using eco-friendly bioprocesses with minimal substrate processing and without any supplementation requirements.

7.2 FUTURE PERSPECTIVES

The results obtained in the present work gave important insights into the development of bioprocesses for enzyme production, especially lipase, by *Yarrowia* species. However, there are some aspects that would be interesting to explore in future work:

- Since *Yarrowia* species are known for the production of several value-added compounds, explore and optimize the production of other bioactive molecules in SSF, for instance, erythritol.
- Scale-up and further optimize the bioprocess stablished for protease production by *Y. lipolytica*.
- Explore SSF with strains from *Y. divulgata*.
- Evaluate the effect of mixing events on lipase and biomass production by *Y. lipolytica* W29 in trays and horizontal drum bioreactors.
- Characterize the fermented solid obtained after SSF with high biomass production (for example, in horizontal drum bioreactor without forced aeration) and evaluate their potential for incorporation in animal feed, such as amino acid profile, protein digestibility and presence of antinutritional compounds.
- Perform a water extraction step before pretreatment of OC to further improve the fractioning of this oil cake.
- Further optimize lipase and biomass production by *Y. lipolytica* using OC hydrolysates based medium, for instance, increase incubation time and evaluate the influence of other culture medium supplementation.