



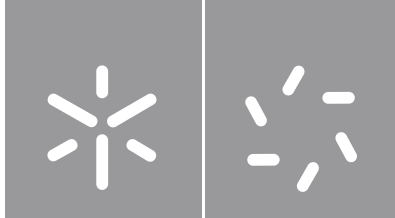
Universidade do Minho
Escola de Ciências

Daniel Filipe Martins Afonso Correia de Sousa

**Agro-industrial wastes up-grading by solid
state fermentation for bioactive
compounds production**

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Escola de Ciências

Daniel Filipe Martins Afonso Correia de Sousa

**Agro-industrial wastes up-grading by solid
state fermentation for bioactive
compounds production**

Doctoral Thesis in Biology

Doctoral Program in Agricultural Production Chains - from fork
to farm

Work developed under the supervision of:

Professor Doctor Isabel Maria Pires Belo

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STATEMENT OF INTEGRITY

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

VALORIZAÇÃO DE RESÍDUOS AGRO-INDUSTRIAIS ATRAVÉS DE FERMENTAÇÃO EM ESTADO SÓLIDO PARA A PRODUÇÃO DE COMPOSTOS BIOATIVOS

A indústria de óleos vegetais tem um papel preponderante nos mercados mundiais devido à importância na alimentação humana e bioenergia e gera grandes quantidades de subprodutos, os bagaços de oleaginosas (BO). Os BO são utilizados principalmente em rações para animais e apresentam diversas características que os tornam substratos adequados para serem utilizados em processos biotecnológicos, para a obtenção de compostos de valor acrescentado.

Neste trabalho estudou-se a fermentação em estado sólido (FES) por três espécies de fungos filamentosos, utilizando o bagaço de girassol (SFC), o bagaço de colza (RSC) e bagaço de soja (SBC). *Aspergillus niger* foi identificado como o fungo que contribuiu para uma maior produção de enzimas e aumento da capacidade antioxidante dos extratos aquosos de BO fermentados, tendo sido selecionado para estudos posteriores de otimização. Apesar do maior potencial demonstrado por *A. niger*, foi estudado o efeito da utilização de consórcios fúngicos, na valorização de SFC e RSC por SSF. O consórcio das espécies, *Aspergillus ibericus* e *Rizophus oryzae*, levou à maior produção enzimática de celulase, xilanase e β -glucosidade. Misturas de RSC e SBC e dos três BO em partes iguais maximizaram a produção enzimática quando fermentados com *A. niger* (299 U g⁻¹ celulase, 1476 U g⁻¹ xilanase, 191 U g⁻¹ β -glucosidase e 220 U g⁻¹ protease). Os resultados obtidos foram reproduzidos com sucesso em reator de tipo bandeja com uma quantidade de substrato até 40 vezes superior. A FES levou a um aumento do conteúdo em proteína bruta, redução da concentração de fibras e de fatores antinutricionais aumentando os coeficientes de digestibilidade. Contudo, também afetou a qualidade da proteína. Após FES verificou-se o aumento do potencial antioxidante dos extratos fermentados em relação aos não fermentados, bem como a diminuição da concentração inibitória mínima contra algumas bactérias, maior inibição do crescimento de fungos fitopatogénicos, e alguns extratos apresentaram efeitos de mitigação de danos da levedura *Schizosaccharomyces pombe* na presença de camptotecina ou bisfenol A. De uma forma geral, os resultados demonstraram o potencial dos BO como substrato para FES, aumentando o seu valor nutricional e produção de compostos bioativos relevantes, com aplicações em indústrias como a alimentar, animal, cosmética ou farmacêutica.

Palavras-chave: alimento animal; bagaços de oleaginosas; bio processamento; bio produtos; compostos bioactivos.

ABSTRACT

AGRO-INDUSTRIAL WASTES UP-GRADING BY SOLID STATE FERMENTATION FOR BIOACTIVE COMPOUNDS PRODUCTION

The vegetable oils industry plays a leading role in world markets due to its importance in human nutrition and bioenergy and generates large amounts of by-products, oilseed cakes (OC). OC are mainly used in animal feed and have several characteristics that make them suitable substrates to be used in biotechnological processes to obtain value-added compounds.

In this work, three species of filamentous fungi were studied in solid-state fermentation (SSF) experiments, using the main OC produced globally, namely sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC)

Aspergillus niger was identified as the fungus that contributed to a greater enzyme production and increased antioxidant capacity of fermented OC aqueous extracts and was selected for further optimization studies. Despite the greater potential demonstrated by *A. niger*, the effect of using fungal consortia was studied for the valorization of SFC and RSC by SSF. The consortium of species, *Aspergillus ibericus* and *Rizophus oryzae*, led to a greater enzymatic production of cellulase, xylanase and β -glucosidase. Mixtures of RSC and SBC and the three OC in equal parts maximized enzyme production when fermented with *A. niger* (299 U g⁻¹ cellulase, 1476 U g⁻¹ xylanase, 191 U g⁻¹ β -glucosidase and 220 U g⁻¹ protease). The results obtained were successfully reproduced in a tray-type reactor with an amount of substrate up to 40 times higher. SSF led to an increase in the crude protein content, a reduction in the concentration of fibers and antinutritional factors and increase of the digestibility coefficients. However, it also affected protein quality. After SSF, there was an increase in the antioxidant potential of the fermented extracts compared to the non-fermented ones, as well as a decrease in the minimum inhibitory concentration against some pathogenic bacteria, greater inhibition of the growth of phytopathogenic fungi, and some extracts showed mitigation effects in the damage of the yeast *Schizosaccharomyces pombe* when in the presence of camptothecin or bisphenol A. In general, the results demonstrated the potential of OC as a substrate for SSF, increasing its nutritional value and production of relevant bioactive compounds, with applications in industries such as food, animal, cosmetics or pharmaceutical.

Keywords: animal feed; bioactive compounds; bioprocessing; by-products; oil cakes.

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LIST OF ABBREVIATIONS

ADF	acid detergent fiber
ADL	acid detergent lignin
ANOVA	analysis of variance
BPA	bisphenol A
BBi	Bowman-Birk inhibitors
CMP	camptothecin
CMC	carboxymethylcellulose
CP	crude protein
CECT	Colección Española de Cultivos Tipo
COVID-19	coronavirus disease 2019
DM	dry matter
DNS	3,5-dinitrosalicylic acid
EC₅₀	effective concentration able to produce 50 % of the maximal response
F/ FER	fermented
F_{EXT}	fermented-extracted
FRAP	ferric reducing ability of plasma
Fe^{III}-TPTZ	ferrous tripyridyltriazine
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GRAS	generally recognized as safe
HPLC	high-performance liquid chromatography
HSD	honestly significant difference
ICA	iron chelating activity
KTI	Kunitz trypsin inhibitors
LSD	least significant difference
LB	lignocellulosic biomass
LF	liquid fermented
NF	non-fermented
NDF	neutral detergent fiber
N	nitrogen
NSP	non-starch polysaccharides

OC	oilseed cakes
OM	organic matter
OECD	Organization for the Economic Co-operation and Development
PNG	<i>p</i> -nitrophenyl- β -D-glucopyranoside
PDA	potato dextrose agar
PCA	Principal Component Analysis
PDI	protein dispersibility index
QAH	quantitative acid hydrolysis
RSC	rapeseed cake
ROS	reactive oxygen species
SSF	solid-state fermentation
SmF	submerged fermentation
SBC	soybean cake
SFC	sunflower cake
SAS	statistical analysis system
TPC	total phenolic compounds
UV/VIS	ultraviolet-visible
WHC	water holding capacity

Remarks:

In general, the International System of Units (SI) was used in this work. Sometimes multiples and sub-multiples 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 the volume percent (% v/v), 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, due to the usual use in fermentation technology area.

SCIENTIFIC OUTPUTS

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.

The results presented in this thesis have been partially published elsewhere.

PEER-REVIEWED JOURNAL ARTICLES

Leite, P.; Sousa, D.; Fernandes, H.; Ferreira, M.; Costa, A. R.; Filipe, D.; Gonçalves, M.; Peres, H.; Belo, I.; Salgado, J. M. Recent Advances in Production of Lignocellulolytic Enzymes by Solid-State Fermentation of Agro-Industrial Wastes. *Curr. Opin. Green Sustain. Chem.* **2021**, 27, 100407.

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SUBMITTED MANUSCRIPTS

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Sousa, D.; Simões, L.; Oliveira, R.; Salgado, J. M.; Cambra-López, M.; Belo, I.; Dias, A. Evaluation of biotechnological processing through solid-state fermentation of oilseed cakes on extracts bioactive potential, submitted to Biotechnology Letters

POSTERS IN CONFERENCES

Sousa, D.; Salgado, J. M.; Dias, A.; Belo, I. **2019** Extraction of bioactive compounds from vegetable oils pomaces. European Biotechnology Congress. Valencia, Spain.

ORAL COMMUNICATIONS

Sousa, D.; Salgado, J. M.; Cambra-López, M.; Dias, A.; Belo, I. **2019** Production of bioactive compounds by solid-state fermentation of oilseed cakes. 5th International Conference - WASTES: Solutions, Treatments and Opportunities. Costa da Caparica, Portugal.

Sousa, D.; Salgado, J. M.; Cambra-López, M.; Dias, A.; Belo, I. **2021** Sunflower and rapeseed cake bioprocessing by fungal consortiums. European Biotechnology Congress. Sofia, Bulgaria.

THESIS OUTLINE

This thesis is divided into 7 main chapters, five of them describing experimental research published or submitted to international peer-reviewed journals.

Chapter I provides a comprehensive review on the major produced by-products derived from the vegetable oils industry. Their production rates and chemical composition are addressed as their applications and role in biotechnological processing for the obtainment of bioactive compounds.

Chapter II focuses on physicochemical characterization of oilseed cakes and their potential as substrate for solid-state fermentation for the production of bioactive compounds. A correlation between each microorganism used in solid-state fermentation and its main bioactivity was demonstrated.

Chapter III explores the use of fungal consortiums of two species in solid-state fermentation for the up-grading of selected oilseed cakes and production of enzymes and antioxidants.

Chapter IV focuses on the optimization of the solid-state fermentation medium by mixing the OC and further scale-up of the process to tray bioreactors.

Chapter V provides a detailed nutritional characterization of oilseed cakes using standard methods for animal feed formulations and evaluates the effect of solid-state fermentation on the nutritional properties of oilseed cakes as well as their *in vitro* digestibility using poultry digestive models. The tasks

displayed in this chapter were performed by the author of this thesis at the Department of Animal Science, Polytechnic University of Valencia (Valencia, Spain).

Chapter VI explores the bioactive potential of oilseed cakes and the effect of solid-state fermentation on bioactive potential of OC extracts. The tasks displayed in this chapter were performed by the author of this thesis at the School of Sciences, University of Minho (Braga, Portugal).

Chapter VII offers the major conclusions of this thesis with empathizes on the biotechnological valorization of oilseed cakes within a biorefinery approach and circular economy concept, alongside future perspectives.

Chapter 1

General Introduction

1.1. Introduction

Sustainable development is a concept that must be rooted in our society. In order to assure life quality for human beings, a more efficient and sustainable use of natural resources must be made. Over the past decades, global population has been increasing at a high rate. Currently, world population is around 8 billion people, and it is expected to reach a peak of 11 billion by the end of this century. This increment leads to a mandatory and continuous search to obtain food. Currently, about 26.4 % of world population, approximately 2 billion people, do not have regular access to safe, nutritious and sufficient food where, 9.2 % are undernourished.¹ Population growth is unavoidable and production chains must be improved, boosting the model of circular economy to reduce food loss and environmental impact of intensive food production. In fact, circular economy is a key factor towards a sustainable development of production patterns.

With respect to human food supply, protein supplementation will be the limiting factor in a near future. In developed countries higher incomes by the families are changing dietary habits leading to an increase of calorie intake and animal protein consumption.² Protein is also present in vegetable sources such as soya, grains, nuts, seeds and certain vegetables.³ However, animal protein is considered a high-quality protein with higher bioavailability of micronutrients such as vitamin A and B-12, riboflavin and minerals such as calcium, iron and zinc.⁴ Protein from animal sources account for 18 % of calorie and 25 % of protein intake worldwide.⁵ Globalization led to a higher world-demand for a variety of foods including meat, leading to an overproduction and consequent generation of food losses. In order to feed animals to supply global animal protein demand, natural resources are constantly under great pressure. The vast majority of arable land is used for the production of grains or oilseeds, used for the obtention of vegetable oils, for biodiesel production and animal feed.² A balanced diet of meat, cereals, oils and vegetables, diminishing meat consumption would decrease the environmental impact caused by this industry.⁶⁻⁹ Additionally, due to the increasing energy demand and environmental problems related with the use of fossil fuels, the production of biofuel arose as a viable alternative. On the other hand, biodiesel is produced from biological sources such as vegetable oils or animal fats.¹⁰ Over the past years, the Human diet has suffered transitions and nowadays, vegetable oils are an unquestionably part of our diet. However, with the increasing demand for food and feed products, vegetable oils production must increase to meet the demand for human consumption and to feed the animals. Although, instead of increasing the pressure on natural resources it is important to take full advantage of these plant sources and use them in biotechnological processes to improve their nutritional properties and obtain value-added compounds.

1.2. Vegetable oils industry

The vegetable oils industry is in continuous expansion. The high demand for vegetable oils and its derivatives has been increasing over the recent years. Despite the stagnations related to the COVID-19 pandemic, followed by the conflict in the Eastern Europe the global markets for oils and fats are set to increase marginally during 2021/22.¹¹ The forecast for the global oilseed production is to reach 604.2 million tons during 2021-2022 season.¹¹ According to the latest Food Outlook from the Food and Agriculture Organization (FAO), Brazil appears as the largest producer of oilseeds (143.1 million tons), followed by the United States of America (USA) (125.1 million tons) and China (62.3 million tons).¹¹ Brazil and the USA are the major producers of soybean seeds while China and Canada (27.1 million tons) are the largest producers of rapeseeds. The European Union (EU) (28.4 million tons) alongside the Russian Federation (Russia) (20.5 million tons) and Ukraine (19.5 million tons) are the major producers of sunflower seeds. According to the United States Department of Agriculture (USDA), these three are the major oilseed crops produced worldwide.¹²

The demand for this commodity is correlated with the high versatility and different applications of vegetable oils. They are primarily used for human consumption although their applications can be extended to the animal feed industry, pharmaceutical and cosmetic industry, for biodiesel production and synthesis of lubricants and other chemical products.^{13,14}

The oil content of oilseeds varies between each species. Soybean seeds are characterized for a low oil content ranging from 18 to 22 % (w/w, dry weight). Other seeds have higher oil content such as rapeseed, canola and sunflower seeds, which may vary between 30 and 48 % (w/w, dry weight) of oil.¹⁵⁻¹⁷ Sunflower seeds contain approximately 35 to 42 % (w/w, dry weight) of oil.¹⁸ Among the available methods used for oil extraction, mechanical pressing and solvent extraction are the most commonly used (**Figure 1.1**).¹⁴ However, prior to oil extraction some pretreatment steps may be required in order to provide an efficient extraction with high yield of oil recovery. Additionally to a first screening and cleaning of the seeds, grinding, heating, cracking and extruding are some pretreatment opinions.^{19,20} Mechanical pressing is considered an easy method for oil extraction since it only requires mechanical power. This process can be performed either using an hydraulic press or an expeller press, also known as extruder or screw press. The main differences between the two types is that screw press can operate in a continuous process and has higher oil recovery yield.¹⁹ During this process seeds are compressed in a container with small perforations on the walls from where the oil leaves the system. Despite the obtainment of a high-quality oil by mechanical pressing this process has a lower yield compared to solvent extraction. A complementary step of solvent extraction can be made after mechanical pressing

with the goal of achieving higher oil recovering yields. During the solvent or chemical extraction of oil from oilseeds, these are in contact with a solvent that dissolves and removes the oil. This method is considered very effective due to the high oil yield and consistence performance.²¹ Hexane is the most commonly used solvent in the oilseed extraction industry. Its efficiency and ease of oil recovery, low cost, recyclability, narrow and low boiling point (63-67 °C) makes this solvent the preferred choice in this

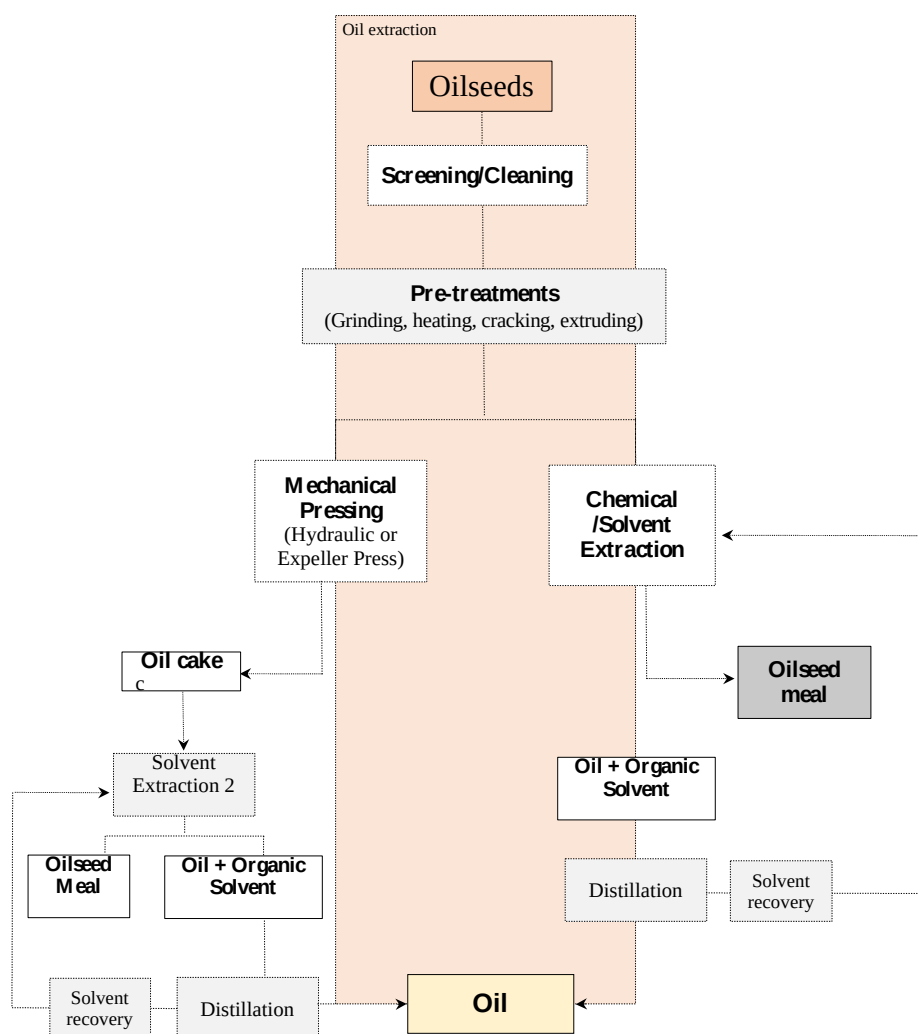


Figure 1.1. Schematic diagram of the vegetable oils extraction process using mechanical pressing and solvent extraction.

industry.^{20,22,23} Although this method has some disadvantages such as the negative environmental impact due to wastewater generation, emission of volatile organic compounds and human health impacts on the workers that manipulate the solvent.²⁴ Independently of the method applied for oil extraction two

direct products are obtained after the process. The extracted oil and a residual solid called cake if obtained by mechanical pressing or meal when the seeds are exposed to solvent extraction.¹⁹

According to the recent OECD-FAO Agriculture Outlook for 2021 - 2030, global crushing of oilseeds accounts for approximately 90 % of total production resulting in a large generation of solid by-products.²⁵

The oilseed cakes (OC) are valuable commodities and commercially traded. About 65 % of produced meals are internationally traded and their price per ton can vary from 300 to 500 USD considering as reference the value of soybean cake over the last years.²⁵

1.3. Production and composition of sunflower cake, rapeseed cake and soybean cake

The method used for oil extraction influences by-products chemical composition. Alongside the extraction method, oil cakes composition may vary depending on the variety of the crop, agriculture management practices and environmental conditions. **Table 1.1** shows the chemical composition of the three major globally produced OC: sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC). These are considered edible OC and they are characterized for their high protein and fiber content. SBC has the highest content of crude protein accounting for approximately 50 % (w/w) on dry matter. This by-product has the lowest content of fiber while SFC has the highest. Fibers of these OC are mainly composed by non-starch polysaccharides (NSP), lignin and non-digestible oligosaccharides which resist to enzymatic digestion.²⁶ Within the NSP structures, cellulose, hemicellulose and pectic polysaccharides are present and most of them are insoluble. Additionally, oligosaccharides can be found in the form of galacto-oligosaccharides. Oil seeds are natural sources of fatty acids and concentration of this compounds in OC is variable, being affected by the extraction method. The main fatty acids found in SFC, RSC and SBC are linoleic acid (18:2), oleic acid (18:1) and palmitic acid (16:0).²⁷⁻²⁹ OC have a balanced content of minerals, which have an important role in enzymatic and metabolic processes, being phosphorus, potassium, calcium, and magnesium the ones with higher concentrations. Additionally, OC contain soluble sugars, vitamins, and fractions of phenolic compounds with bioactive properties.³⁰ However, improper handling and storage conditions may affect the nutrients properties as well as organoleptic properties such as taste and smell.³¹

Table 1.1. Chemical composition of major oilseed cakes

Parameter (g kg⁻¹)	Sunflower cake	Rapessed cake	Soybean cake
Moisture	5.56 – 9.82	6 – 15.3	8.4 – 13.2
Dry matter	90.18 – 94.44	84.7 – 94.3	86.8 – 91.6
Ash	4.56 – 8.38	5 – 19.7	5.71 – 6.26
Crude Protein	19.93 – 40.20	14.04 – 40.1	40.2 – 50.4
Lipids	2.44 – 31.42	2.04 – 23.1	1.72 – 20.50
Crude Fiber	18.4 - 31.88	5.5 – 15.46	4.95 – 11.28
Minerals (g kg⁻¹)			
Calcium	4.2	8.2	3.3
Phosphorus	10.6	11.9	6.2
Magnesium	5.2	5.3	2.6
Potassium	15.4	12.5	20.2
Sodium	0.08	0.56	0.05
Chlorine	1.4	0.8	0.3
Sulfur	3.3	8.3	3.2
Minerals (mg kg⁻¹)			
Manganese	45.0	63	30
Zinc	94.0	64	50
Copper	32.0	5	15
Iron	496	187	119
Selenium	0.3	0.2	0.3
Cobalt	0.2	0.1	0.1
Molybdenum	2.0	2	4
Iodine	0.1	0.1	0.1
Amino acids (g kg⁻¹)			
Arginine	29.2	17.7 – 19.6	29.4
Cysteine	6.1	7.2 – 8.1	5.9
Glycine	20.3	15.6 – 16.7	17.3
Histidine	8.6	7.8 – 9.1	11
Isoleucine	14.7	11.9 – 13.6	18.7
Leucine	22.0	20.9 – 23.0	30.3
Lysine	12.9	17.0 – 18.4	25
Methionine	8.1	5.9 – 6.8	5.8
Phenylalanine	15.8	11.7 – 13.0	20.3
Threonine	13.0	13.4 – 14.7	16.3
Tryptophan	4.6	4.2	5.1
Tyrosine	8.4	9.6	14.3
Valine	17.6	15.6 - 17.3	19.4
Alanine	15.4	13.2 – 14.7	17.1
Aspartic acid	31.5	21.8 – 24.0	44.8
Glutamic acid	68.4	47.0 – 57.0	70.8
Proline	15.0	17.5 – 20.4	20.2
Serine	14.9	12.5 – 14.7	21.2
References			
	29,32,42–45	2,28,34,43,44,46	29,34,43,47

The presence of small quantities of water and readily available carbon sources may promote the growth of microorganism's which may lead to contamination of OC. To prevent contamination, OC are first air-dried before storage, and they can be found as flour or as pellets.³² Due to their chemical composition, OC found several applications which may include bio-fuel production, animal feed, organic fertilizers, and landfill. The use of OC as animal feed can be limited by seasonal production, low levels of nutritive components, due to the presence of toxic compounds or even by changes in nutritional composition which may affect diet formulation.³¹ OC and more precisely soybean derived by-products are mostly used for animal feed. SBC is primely used in the diets of monogastric species (chickens and pigs) while RSC and SFC are mostly used in meals for pigs.³³

Another application of OC in animal feed is as ingredients of fishmeal.^{34,35} RSC and SFC present some limitations regarding their incorporation in animal feed formulations due to the high content of indigestible fibers which may reduce digestibility or due to the presence of antinutritional factors that can be harmful to animals.³⁶⁻³⁸ Thermal treatments during oilseeds processing may reduce some of these factors but it may also result in partial denaturation of proteins which will affect their structure and functional properties as well as their digestibility.^{39,40} The decomposition of residues in landfill may led to the production of methane due to the presence of heavy metals or organic compounds.⁴¹ The process of bio-fuel production can be achieved either by anaerobic digestion which is more directed for products with high organic and water content or by a thermochemical process which include pyrolysis, combustion, gasification or even incineration which has high economic costs and entails environment problems.^{31,48,49} OC can be used as fertilizers as they are natural sources of N and they can be used for the slow release of N as well as to provide organic matter to soils minimizing the effects of intensive agriculture.⁵⁰⁻⁵³ Despite OC applications there are other strategies to valorize them within the concept of circular economy. OC can be used for the extraction of bioactive compounds which include protein isolates, antioxidants, or dietary fibers. Also, OC can be used for the development of new food products through their incorporation. Possible uses of high protein and fibrous OC can be found in the bakery or confectionary industry for the development of new breads, biscuits, snacks or even desserts.³¹ An alternative way of capitalizing OC valorization is through the use of biotechnological processes for the production of functional ingredients which have important benefits to consumers.⁵⁴ Among the possible products obtained from biotechnological processing of OC are included enzymes, antibiotics, biopesticides, aroma compounds, pigments, vitamins or biosurfactants and mushrooms. OC have a tremendous potential for being used as substrate in biotechnological process due to their high availability and nutrient composition that require small or even no supplementation.

1.4. Oilseed cakes as animal feed

Oilseed processing by-products have been routinely used as animal feed or included in animal feed formulations. Feeding by-products to animals is a common practice and in the USA the acceptance of OC as part of the diets of livestock and poultry remounts to the mid-1930's.⁵⁵ SBC or soybean meal is generally regarded as one of the best sources of protein available for animals and it is the standard to which other protein sources are compared.⁵⁶ In the 2019-2020 season, rapeseed and sunflower meal were the major sources of plant proteins, after soybean meal, used to feed livestock in the European Union.²⁶

In order to achieve optimal production, animals require the right amount of nutritionally adequate feed. A diet based on a mixture of various raw materials is essential to achieve high quality livestock products (meat, milk, and eggs) and for a healthier animal growth.

Edible OC are safe and nutritious sources of protein with appropriated quantities of carbohydrates and fats to provide energy for animals. Additionally, they contain minerals and vitamins essential for proper body functions. Of all the legume seeds, SBC has the highest protein content and has a balanced amino acids profile.⁵⁷ Its amino acids are highly digestible (more than 90 % in pigs and poultry) and contain high amounts of lysine, tryptophane, threonine and isoleucine which are often in deficit in cereals grains.⁵⁸ However, the cysteine and methionine concentrations are bellow optimal standards for monogastric animals but this deficiency can be easily overcome by the addition of synthetic amino acids.^{39,59} Methionine is the first limiting amino acid and is of particular importance in high energy diets. Despite the advantages of this protein source, SBC contain several substances that can have a toxic, stimulative, or inhibitory effect.⁵⁶ Among these substances and of particular importance considering animal nutrition are trypsin (protease) inhibitors. The trypsin inhibitors are of two main types: the Kunitz trypsin inhibitors (KTI) and the Bowman-Birk inhibitors (BBI). The KTI are considered larger and stronger inhibitors of trypsin while the BBI are considerably smaller and are able to inhibit both trypsin and chymotrypsin.⁶⁰ The protease inhibitors present in SBC negatively affect the activity of proteolytic endogenous enzymes.⁶¹ Proteases are enzymes able to digest both stored proteins as well as proteinaceous antinutrients present in vegetable proteins.⁶² The adverse effect of these inhibitors on reducing the activity of the digestive enzymes trypsin and chymotrypsin is correlated with inhibition of protein digestion and consequent growth retardation.^{56,63} Fortunately, trypsin inhibitors are easily destroyed by heat treatment.³⁹ Although other factors considered as relatively heat stable, despite being in smaller concentrations are present in SBC and may impair nutrients utilization. These factors include goitrogens, tannins, non-starch polysaccharides and phytate.⁶³ Goitrogens and tannins are natural

occurring substances and they are considered as undesirable substances in animal feed as they may interfere with iodine uptake affecting the function of the thyroid gland (goitrogens) or in the case of tannins may precipitate with carbohydrates or proteins to form enzyme resistance substrates, inhibit digestive enzymes or interfere with minerals and vitamins bioavailability.^{56,64,65} The presence of non-starch polysaccharides (NSP) is a transversal factor to all OC as they alongside lignin are the primary constituents of plant cell walls and comprise up to 20 % of plant-based poultry diets.⁶⁶ The presence of these factors is mainly a drawback considering poultry feed as they lack on NSP-digestive enzymes.⁶⁷ Additionally, NSP depress the digestibility of protein, starch and fat.⁶⁸ The presence of these polysaccharides increases microbial fermentation which may cause intestinal disorder. Phytate or phytic acid is the main storage form of organic phosphorus in plants seeds. This element is an essential and limiting nutrient in animal feed. Despite the high content of P in SBC much of it is in the form of a phosphorus-phytate complex but it is a poorly digestible form for monogastric animals.⁵⁸ When not digested, the ingested P is then excreted in the manure which can raise concerns regarding the effects of P on eutrophication.⁶⁹ Additionally, phytic acid is a strong chelation agent that can bind to several mineral cofactors affecting digestive proteins.⁷⁰

The presence of antinutritional factors is not exclusive of SBC. RSC and SFC also contain antinutritive factors that limits their applications in animal feed formulations. In fact, the inclusion of these OC in feed formulations is defined for a maximum of 10 and 11 %, respectively for poultry feed formulations while for cows the incorporation of RSC and SFC is respectively 26 and 25 %.⁷¹ This inclusion rates are attributed to the higher fiber content of these OC in comparison to SBC as well as the lower digestible protein and lysine contents and the presence of antinutritional factors.^{58,72,73} RSC is characterized for the presence of glucosinolates (thioglucosides), erucic acid and tannins as its main antinutritional factors.⁵⁶ Glucosinolates are sulfur-containing plant secondary metabolites present in all Brassica-originated feeds. They are present in all plants part and are physically separated from the enzyme myrosinase (thioglucoside glucohydrolase).⁷⁴ Under several conditions, glucosinolates are hydrolyzed by the myrosinase present in plant and by the same enzyme produced in the intestinal microflora.^{75,76} Isotiocyanates, organic thiocyanates, nitriles and 5-vinyloxazolidine-2-thione (goitrin) are products of glucosides breakdown.⁵⁶ The toxic effects induced by these derivatives can be manifested as goitres and liver and kidney poisoning. The adverse effects of these compounds are more pronounced in non-ruminant animals. In poultry their ingestion resulted in increased mortality and lowered egg production as well as egg weights while in pigs, meals with glucosinolates may reduce food intake, growth and loss of carcass quality.^{56,74} Erucic acid causes a bitter taste and is responsible for adverse effects on heart

health and animal performance.^{56,77} Despite the severe effects of these by-products, the development of rape cultivars with low erucic acid and low glucosinolates content potentialized the inclusion rates of these products in animal feed formulations. Notwithstanding that the presence of this antinutritional factors is considerably lower they should not be underestimated. Apart from the undesirable effects of antinutritional factors present in RSC this OC has a balance of amino acids that compares favorably to that of SBC despite RSC having lower lysine but more methionine. Additionally, the balance of calcium and phosphorus is satisfactory having a higher phosphorus content than other oilseed by-products.⁵⁶

One of the main traits of SFC is the absence of intrinsic antinutritional factors comparing to other OC. Also, it has a richer amino acid profile in sulfur-containing amino acids than other OC, in special methionine.⁷³ However, its lysine content is considerably lower than that of SBC. Additionally, the presence of a high fibrous fraction in SFC, its low metabolized energy as well as the presence of polyphenolic compounds mainly chlorogenic acid restricts its use in animal and poultry feed.⁷⁸

These OC are recognized vegetable nutritious protein sources that have been used in the animal feed industry. Animal feed supplementation with exogenous enzymes (carbohydrase's, protease, phytase) is a common practice to overcome some of the limitations imposed by the higher fiber content of some OC or the presence of antinutritional factors.⁷⁹⁻⁸² Although, the transformation of these OC through biotechnological valorization of raw materials may entail additional benefits to their nutritional value with possible decrease of antinutritional factors increasing their potential in the feed industry.

1.5. Role of oilseed cakes in biotechnology processes

The biotechnology industry has a preponderant role on the reutilization and valorization of wastes or industrial by-products to obtain value-added products. Through the use of green, low-cost, and low-environmental impact biotechnological processes it is possible to contribute for the development of a bioeconomy based on the use of renewable resources contributing to sustainable production chains.

OC have been the focus of several biotechnological approaches. Their chemical composition combines the perfect environment for microbial growth. OC are able to provide both carbon and nitrogen sources and, therefore, they are suitable substrates for being used in the biotechnological industry to obtain value added compounds.^{43,83} The main applications of OC in biotechnological processes have the final purpose of producing industrial enzymes, antibiotics and biopesticides, antioxidants, vitamins, edible mushrooms, biogas, and bio-oil.⁸⁴ The production of bioactive compounds using OC as substrate in biotechnological processes can be achieved either by submerged fermentation (SmF) or by solid-state fermentation (SSF) (**Figure 1.2**).

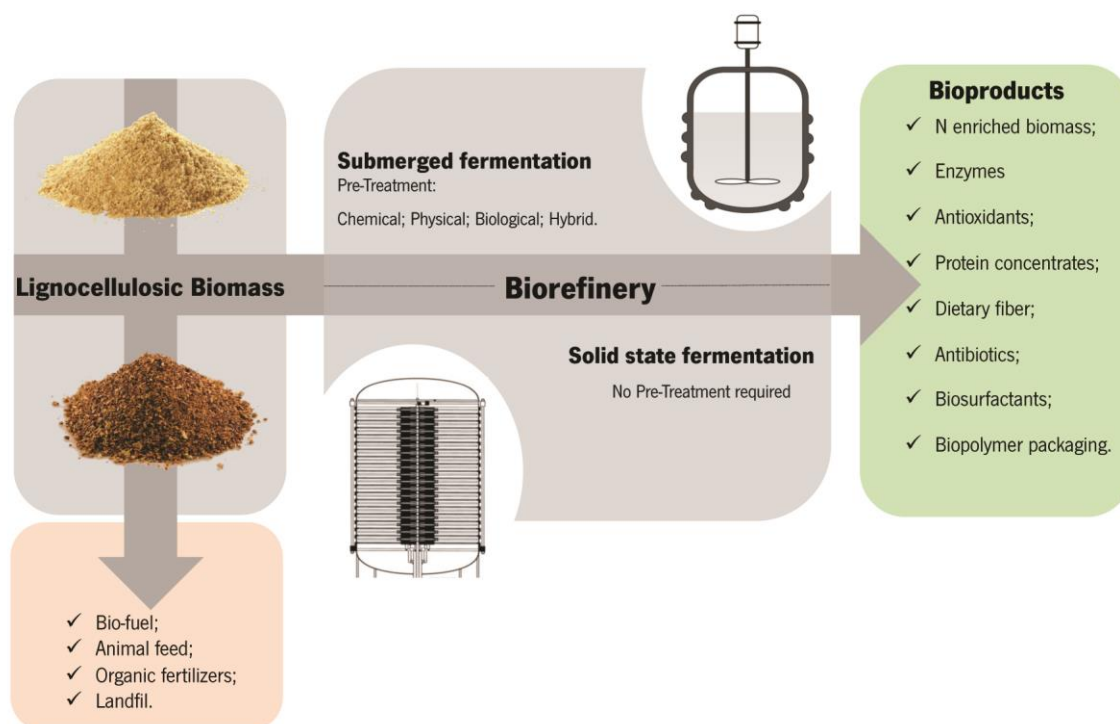


Figure 1.2. Biorefinery scheme for valorization of lignocellulosic biomass.

SmF is a process where microorganisms grow in liquid mediums optimized with the required nutrients for microbial growth. However, in this type of fermentation solid by-products cannot be directly applied into the fermentative medium. Due to the natural intermeshed plant cell wall composition, that includes cellulose, hemicellulose and lignin, forming a complex recalcitrant structure, the use of lignocellulosic by-products in this type of fermentation requires pre-treatment steps that will fractionate the crystalline structure.⁸⁵ Pre-treatments can be classified into chemical (alkaline, acid, ionic liquids), physical (mechanical, ultrasonic, hydrothermal), biological (fungi or bacteria, enzymes) or an hybrid method.⁸⁶ Acid hydrolysis, autohydrolysis or enzymatic hydrolysis are examples of pre-treatments that will contribute to the disruption of the lignocellulosic structure, exposing polysaccharides to the action of cellulolytic enzymes and consequent release of simple fermentable sugars, after enzymatic hydrolysis.⁸⁷ The pre-treatment process will breakdown by-products structure resulting in a number of components including proteins and carbohydrates that will compose a suitable fermentative medium for the production of industrial interesting compounds.⁸⁸

On the contrary, SSF is a process in which microorganisms, in single or mixed cultures, grow on solid substrates in an environment with null or almost null free water content. Filamentous fungi are the microorganisms that better adapt to this type of fermentation, but yeast and bacteria have been also successfully employed in SSF. This type of fermentation has several advantages comparing to SmF

such as less production of wastewater with less risks of contamination by bacteria, fermentation time may be shorter, requires less water input and overall it is a more cost-effective and eco-friendly process.^{89,90} Additionally, by-products can be directly used as substrate in SSF, without any pre-treatment step. Some of the diverse range of applications of OC in the referred SSF bioprocess are summarized in

Table 1.2.

Among the wide biotechnological applications of OC, the production of industrial enzymes is perhaps the most significant. The market of enzymes for industrial applications is continuously growing and is expected to reach 7.0 billion by 2023, which is an increase of approximately 27 % comparing to the year of 2018.⁹¹ The production of microbial enzymes has several industrial applications including the textile, food, animal feed, paper, biofuel and bioethanol industries.⁹²⁻⁹⁴ OC composition favors the production of microbial enzymes in biotechnological process due to their lignocellulosic nature. The lignocellulosic fractions can act as inductors or inhibitors for the production of lignocellulolytic enzymes.⁹⁵ The use of OC has been reported for the production of several classes of enzymes including carbohydrase's (xylanase, cellulase, β -glucosidase, α -amylase), phytase, protease and lipase.

Rekha et al.⁹⁶ used groundnut oil cake in SSF for the production of lipase using *Candida rugosa*. Ramachandran et al.⁹⁸ reported the production of α -amylase using coconut oil cake as substrate for SSF using the fungus *Aspergillus oryzae*. Enzymatic cocktails composed by xylanase, cellulase, β -glucosidase and protease were reported by Sousa et al.⁴³ using SFC, RSC and SBC as individual substrates for SSF with the fungi *A. niger*, *A. ibericus* and *Rhizopus oryzae*. Maghsoodi et al.¹⁰⁵ reported the production of an alkali protease using *Bacillus licheniformis* in SmF with soybean meal. Phytase has been produced using groundnut oil cake and sesame oil cake through SSF using *Mucor racemosus*.¹⁰⁸

Table 1.2. Oilseed cakes applications in biotechnological process and generated products.

Oil by-product	Process	Microorganisms	Product	Reference
Groundnut oil cake	SSF	<i>Candida rugosa</i>	Lipase	96
Linseed oil cake and mustard oil cake	SSF	<i>Pseudomonas aeruginosa</i> LB-2	Lipase	97
Coconut oil cake	SSF	<i>Aspergillus oryzae</i>	α -amylase	98
Sunflower cake, rapeseed cake, soybean cake	SSF	<i>A. niger</i> , <i>A. ibericus</i> , <i>Rhizopus oryzae</i>	Cellulase, xylanase, β -glucosidase, protease	43
Linseed cake	SSF	<i>Mortierella alpina</i>	Polyunsaturated fatty acids	99
Coconut oil cake, groundnut oil cake	SSF	<i>Amycolatopsis Mediterranean</i>	Rifamycin	100
Cotton seed meal	SSF	<i>Streptomyces fradiae</i> NCIM 2418	Neomycin	101
Peanut press cake	SSF	<i>A. awamori</i>	Antioxidants	102
Groundnut oil cake, coconut oil cake	SmF	<i>R. oryzae</i> MTCC 8784	Lactic acid	103
Soybean meal, cotton seed meal	SmF; SSF	<i>Bacillus licheniformis</i> ; <i>A. niger</i>	Protease	104,105
Olive pomace	SSF	<i>Yamadazyma guilliermondii</i> , <i>Yarrowia lipolytica</i> ; <i>Xanthophyllomyces dendrorhous</i> , <i>Sporidiobolus salmonicolor</i>	Astaxanthin (pigment)	106
Rapeseed cake	SmF	<i>Bacillus amyloliquefaciens</i> CX-20	Iturin A	107
Groundnut oil cake, sesame oil cake	SSF	<i>Mucor racemosus</i>	Phytase	108
Karanja, jatropha	SSF	<i>Paecilomyces variotii</i>	Dipicolinic acid	109
Mustard oil cake sunflower oil cake	SSF	<i>Pleurotus sajor-caju</i>	Mushrooms	110

Besides enzymes production, OC have been reported for their use in the production of antibiotics, biopesticides and pigments. The production of the antibiotic rifamycin has been reported by Vastrad and Neelagund,¹⁰⁰ using coconut and groundnut oil cakes as SSF substrates using *Amycolatopsis Mediterranean*. The same authors reported the production of neomycin in a different study, using cotton seed meal as substrate for SSF with *Streptomyces fradiae* NCIM 2418.¹⁰¹ The production of Iturin A by

SmF of rapeseed meal with *Bacillus amyloliquefacien* has been described by Chen et al.¹⁰⁷ The biocontrol agent dipicolinic acid has been produced through SSF of Karanja and Jatropha oil cakes with the specie *Paecilomyces variotii*.^{109,111} The bioprocessing of olive pomace through four different yeast species (*Yamadazyma guilliermondii*, *Yarrowia lipolytica*, *Xanthophyllomyces dendrorhous*, *Sporidiobolus salmonicolor*) allowed the optimization of the production of the astaxanthin pigment.¹⁰⁶

The range of bioactive compounds produced by exploitation of OC through biotechnological processes is diverse and allowed the obtainment of other commercially value products. Ferreira et al.⁹⁹ reported the valorization of linseed oil cake through its increment in polyunsaturated fatty acids through SSF of this cake with *Mortierella alpina*. The production of lactic acid is another positive result of OC biotechnological exploitation. Ranjit et al.¹⁰³ reported the production of this acid through SmF of groundnut and coconut oil cakes using *R. oryzae* MTCC 8784. The bioprocessing by SSF of OC was also reported by several authors to obtain phenolic extracts with increased antioxidant activity.^{43,102,112}

Oilseeds are natural sources of bioactive phytochemicals being phenolic compounds the most notably produced.¹¹³ Phenolics are naturally produced within plants as secondary metabolites and they play a significant role in many physiological and metabolic process.^{114,115} The structure of these compounds consists of one or more hydroxyl groups attached to an aromatic ring and their structure can be simple or highly complex.¹¹⁶ Phenolics or polyphenols can be classified in several classes including phenolic acids, flavonoids, stilbenes, coumarins, lignans and tannins.¹¹³ Phenolic acids are the most widely distributed phenolic compounds and combine hydroxybenzoic acids (C_6-C_1) including gallic acid, p-hydroxybenzoic, syringic acid, salicylic and vanillic acids as well as hydroxycinnamic acids (C_6-C_3) such as chlorogenic, cinnamic, caffeic, p-coumaric, ferulic and sinapic acids.^{117,118} Flavonoids are composed by a structure of 15 carbons with a common three ring structure in the $C_6-C_3-C_6$ form and are divided into six main classes including flavones, isoflavones, flavanones, flavonols, flavanols, and anthocyanins.¹¹⁸⁻¹²⁰

Stilbenes are characterized for containing two benzene rings bounded by an ethanol or ethylene molecule and this class include compounds such as resveratrol.^{118,121} Coumarins result from the association of a benzene with a α -pyrone ring and can be found in a variety of plant sources in the form of benzopyrene derivatives.^{122,123} Additionally, phenolics can be found in the polymeric form in plants. Lignin is a polymer of monolignols being the three most important p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These are incorporated in the lignin macromolecule as p-hydroxyphenyl, syringyl and guacyl.¹²⁴ Tannins are phenolic polymers and exist as hydrolysable tannins including ellagitannins

and in the condensed form known as proanthocyanidins which are polymers of catechin and epicatechin.^{118,125}

Phenolic compounds can be found either in the soluble or insoluble form.¹²⁶ This distinction is based on their association to the plant cell wall and solubility in aqueous-organic solvents. Soluble phenolics can be found in a free or in a conjugated form, esterified or covalently linked to fatty acids, sugars or small peptides.¹²⁷ On the other hand, insoluble phenolics can be found associated with plant cell wall macromolecules such as pectin, cellulose, hemicellulose and structural proteins through covalent bounds.¹¹⁸

Phenolic compounds protective effect is normally attributed to their antioxidant potential which mitigates the oxidative damage induced by reactive oxygen species (ROS) as response to environmental stress.¹¹⁴ They can act as direct antioxidants and their abilities include radical scavenging, recombination of radicals, reduction potential and chelation of metal ions. Additionally, phenolic compounds can act as indirect antioxidants through the induction of endogenous protective enzymes.¹²⁸ Generally, during oilseeds processing liposoluble phenolic antioxidants are extracted into the crude oil and can be recovered from a deodorization concentrate. However, more polar phenolic compounds remain in the OC.¹²⁹ The phenolic fraction present in OC can vary depending on the oilseed variety, extraction method and growing conditions.¹²⁹ Although, these fractions include various chemical structures such as tocopherols, carotenoids, flavonoids, lignans, phenolic acids and tannins.¹²⁹

The most relevant phenolic compounds and the corresponding phenolic class present in SFC, RSC and SBC are represented in **Table 1.3**. Sunflower and rapeseed are rich in phenolic acids. Chlorogenic acid alongside caffeic acid are the main phenolic compounds found in sunflowers. Chlorogenic acid contributes up to 2.8 % of dry matter while caffeic acid is present in lower concentrations.¹³⁰⁻¹³² Sinapic acid is the main form of phenolic acids found in rapeseed accounting for approximately 80 % of total phenolic compounds.¹³² In lower quantities *p*-coumaric and trans-ferulic acid are other phenolic acids that can be found in RSC. Tannins are another class of phenolic compounds that can be found in RSC (up to 3 %).¹³³ On the other hand, soybean has low content of phenolic acids (*p*-hydroxybenzoic, ferulic, *p*-coumaric, caffeic acids) but has high concentration of isoflavones, particularly genistein and daidzein, which is a subfamily of flavonoids.^{130,134}

Phenolic compounds may interact with proteins leading to the formation of protein and phenolic compounds complexes.¹³⁵ The formation of these complexes can decrease the nutritive value of protein as result of alterations of proteins digestibility, bioavailability, functionality and self-life stability.^{136,137}

Additionally phenolic fractions may interfere with organoleptic properties such as a change to a darker color due to phenolics oxidation.¹³⁸

Table 1.3. Main phenolics in oilseed cakes.

By-product	Main class of phenolic compounds	Phenolic compounds	Reference
Sunflower cake	Hydroxycinnamic acid	Chlorogenic acid (80 % of total phenolic compounds), caffeic acid, cinnamic acid	129,139–141
Rapeseed cake	Hydroxycinnamic acid	Sinapic acid, <i>p</i> -coumaric, ferulic acid, vanillic acid, syringic acid	129,142–145
Soybean cake	Isoflavones	Genistein, daidzein	146,147

In contrast to the previously described undesirable effects of phenolic compounds, these have been the focus of many research studies due to their health-promoting properties including reduced incidence of chronic diseases such as type-2 diabetes, obesity and cardiovascular disease and, anti-inflammatory, antimicrobial and anti-mutagenic properties.^{113,130} Several studies have shown the high antioxidant potential exhibited by numerous phenolics including caffeic, chlorogenic and ferulic acids.^{148–153} Sinapic acid and its derivatives demonstrated antioxidant, antimicrobial, anti-inflammatory, anticancer and anti-anxiety activities.^{154–157} Additionally to antioxidant, antimicrobial and antitumoral activities, soybean isoflavones major importance is as phytoestrogens.¹⁵⁸ Also, their intake has been associated with reduced risk of hormone-like cancers.^{159,160} These phenolic compounds find applications in several industries including the pharmaceutical, cosmetics, food, textile and packaging industries.¹⁶¹

OC are an unquestionably natural source of phenolic compounds with a wide range of bioactivities and applications. Even though, they undoubtedly have the potential to be further exploited for additional obtainment of bioactive compounds with focus on the improvement of production chains through maximization of their potential. Despite the extraction of several phenolic forms some still remain connected to the lignocellulosic structure of OC and biotechnological process can be the solution to increase their recovering yield.

1.6. Challenges and further perspectives

OC are important sources of proteins, dietary fibers, fats, minerals, vitamins, and bioactive antioxidant compounds. Their application in a variety of industries is unquestionable as well as the potential health promoting effects of their derivative compounds. Although further studies regarding maximization of their potential should be made. The OC present limitations regarding their incorporation in animal feed, attributed to the presence of antinutritional factors and high fiber. These are limiting factors when considering the application of OC in animal feed. Antinutritional factors can be harmful to animals well-being while high fiber content may limit digestion. Biotechnological processes, more precisely solid-state fermentation, has proven to be a viable technique for the valorization of by-products and even wastes transforming them into value-added products with new market applications. In this sense, the exploitation of OC using biotechnological process may help to overcome these limitations increasing the potential applications of these natural proteinaceous sources.

It is essential to reuse by-products in order to increase their applications and obtain maximum profit of their characteristics within the concept of circular economy and the biotechnological valorization of these by-products is a reliable procedure with low environmental impact.

1.7. Objectives

Production chains improvement is the key for a more sustainable and green development minimizing the environmental impact through by-products valorization, creating new workplaces and business opportunities. Oilseed cakes (OC) and meals have great potential to be valorized boosting their application as more efficient feed ingredients, as well as feedstock to new biotechnological processes. Although, the main OC application is in the animal feed sector, there are still some limitations due to the presence of antinutritional factors. The biotechnological valorization of OC for monogastric animals feed may lead to improved feed utilization, with economic benefits to the feed and animal production sectors, and simultaneously reducing the pressure on natural resources through higher incorporations of OC in the feed. In addition, biotechnological OC processing originates the production of bioactive compounds with industrial applications and high economic value. Thus, the main goal of this work was to demonstrate the potential of SSF as a biotechnological approach to treat OC, leading to added-value new products.

The SSF processing of OC is a ecofriendly technology that is in accordance with global Sustainable Development Goals (SDGs), particularly SDG 12 ensuring sustainable production patterns. Also, this

work intended to contribute to the SDG 2 (zero hunger), SDG 8 (economic growth) and SDG 13 (climate action). Therefore, the following major objectives were outlined in the present work:

- Selection of the filamentous fungi species suitable to ferment the OC;
- Characterization of the bioactive products obtained from OC fermentation;
- Demonstration of the impact of SSF on the OC composition and their application in animal feed;
- Exploitation of the bioactivities of extracts from fermented OC.

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Chapter 2

Degradation of lignocellulosic matrix of oilseed cakes by solid-state fermentation: fungi screening for enzymes production and antioxidants release

This chapter is based on the following original research article:

Sousa, D.; Salgado, J. M.; Cambra-López, M.; Dias, A. C. P.; Belo, I. Degradation of Lignocellulosic Matrix of Oilseed Cakes by Solid-State Fermentation: Fungi Screening for Enzymes Production and Antioxidants Release. *J. Sci. Food Agric.* **2021**. <https://doi.org/10.1002/jsfa.11490>.

Abstract

Vegetable oils are yearly produced in large amounts generating solid by-products, the oilseed cakes (OC). OC are lignocellulosic materials that have been used for animal feed with some limitations due to high fiber content from the plant cell walls. Biotechnological processes can help to overcome these limitations and contribute to up-grading such by-products, enhancing their nutritional value as feed ingredients.

All fungal species were able to decrease neutral detergent fiber and acid detergent fiber in all by-products. Additionally, relevant enzymes were produced by the three fungi studied resulting in an improved antioxidant capacity of all fermented OC. *Aspergillus niger* led to the highest activity of cellulase (109 U g⁻¹), xylanase (692 U g⁻¹) and protease (157 U g⁻¹) per dry OC matter and to the recovery of an extract rich in antioxidants, with the highest scavenging potential of free radicals and superoxide anion, iron chelation ability and reducing power. *Rhizopus oryzae* produced the highest activity of b-glucosidase (503 U g⁻¹) and led to the highest liberation of total phenolic compounds (TPC). Principal components analysis showed that extracts with high antioxidant potential were obtained in SSF with high enzymatic activity. A positive correlation was established between the action of b-glucosidase and TPC.

Within the same bioprocess it was possible to improve the nutritional value of oilseed cakes and to obtain relevant bioactive compounds such as lignocellulosic enzymes and phenolic compounds with antioxidant potential, resulting in a significant improvement of already valuable by-products with commercial interest for animal feed.

Keywords: sunflower cake, rapeseed cake, soybean cake, lignocellulolytic enzymes, phenolic compounds, filamentous fungi

2.1 Introduction

According to the latest report of the United Nations, it is expected a global population of 9.73 billion people by 2050 and 11.2 billion people by the end of the century,¹ implying an increase for food demand and of overall food production.

Vegetable oils are an important part of the Human diet as they constitute one of the main sources of calories and energy. Hence, they have an unquestionable role in our daily lives due to their versatility and multiple applications in areas such as the food industry, cosmetics, pharmaceutical and also in the biofuels production.² Soybean (*Glycine max*), rapeseed (*Brassica napus*) and sunflower (*Helianthus annuus*) are the three main sources of oilseed for vegetable oils production. According to the United States Department of Agriculture for the 2020/2021 season, global production of soybean, rapeseed and sunflower are expected to reach 361.08, 68.73 and 50.14 million metric tons.³

Extraction of vegetable oils entails the production of by-products, and the main ones are solids normally referred as cakes (residue obtained after screw press) or meal if it has suffered any additional process, usually organic solvent de-oiling process. These are characterized by their high protein content and high fraction of structural compounds from plant cell walls including cellulose, hemicellulose, pectin and lignin.⁴

Oilseed cakes (OC) are traditionally used for animal feed or plant/soil compost. These two options are seen as economically valuable and profitable ways of disposing by-products without any additional cost and with minimum environmental impact. The abundance of protein, carbohydrates and minerals make these by-products suitable to animal feed. However, relatively high amounts of indigestible fiber fraction and lignin,⁵ may hinder nutrient digestibility in animals.

Due to the volume of OC produced every year and their composition, it is important to explore alternatives that can add value to these by-products, using biotechnological and clean processes. In fact, the chemical composition of OC, combine the perfect environment for microbial growth. Therefore, it is essential to find eco-friendly approaches to achieve an adequate valorization of these by-products, such as biotechnological processes that may lead to the obtainment of value-added compounds, decreasing environmental impact and creating new business opportunities and workplaces, contributing to the implementation of circular economy in agro-food industries.

OC are lignocellulosic materials, suitable to be used as substrate in solid-state fermentation (SSF). SSF is defined as a process that occurs in the absence or near absence of free water using a natural or inert substrate as solid support and ensuring enough moisture to support the growth and metabolism of

microorganisms. This fermentation mimics conditions close to the natural habitat of many microorganisms of which filamentous fungi are the ones that better adapt to SSF.⁶

Sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC) have high protein content (300 to 500 g Kg⁻¹) and total carbon ranges between 450 and 500 g Kg⁻¹. Lignocellulosic fractions of these by-products can act as inducers or inhibitors of the production of extracellular enzymes in SSF, depending on their concentration.⁷ This complex matrix is recalcitrant to degradation due to the covalent bonds between hemicellulose and lignin established by hydroxycinnamic acids such as ferulic acid.⁸ Phenolic compounds can be found within the matrix of plant cells in different forms: free phenolics, soluble phenolics (conjugated) or insoluble phenolics. Soluble phenolics are normally esterified to soluble compounds with low molecular weight such as carbohydrates, proteins or lipids.⁹ Insoluble phenolics are also known as non-extractable phenolics and remain in the matrix of the residues after extraction of soluble phenolics. These compounds can be found within the cell wall matrix of vegetable cells and can be released by enzymes.¹⁰ Phenolic compounds have potential health benefits due to their antioxidant properties such as electrophilic scavenging, inhibitors of reactive oxygen species (ROS) and metal ions chelation. Phenolic acids and flavonoids are among the major families of antioxidants present in SFC, RSC and SBC.¹¹ Extraction of antioxidant compounds allows their incorporation in industries such as food being used as food additives or functional foods,¹² in the pharmaceutical industry¹³ once these compounds can reduce the excessive accumulation of free radicals in the body decreasing the prevalence of chronic diseases and they can also have applications in the cosmetics industry for the formulation of anti-aging products.¹⁴

Bioprocessing of SFC, RSC and SBC by SSF allow the liberation of bioactive compounds with potential antioxidant activity, improving their bioavailability. Additionally, SSF allow the production of extracellular hydrolytic enzymes with commercial interest including as supplement in animal feed.¹⁵

This work describes the use of by-products from the vegetable oils industry, SFC, RSC and SBC as substrate for SSF using three filamentous fungi species. Enzymes, such as cellulases and xylanases were monitored in water extracts of the fermented cakes, as well as total phenolic compounds (TPC) and antioxidant potential. In addition, fermented oilseed cakes were characterized to evaluate their nutritive potential to be used in animal feed.

2.2 Materials and methods

2.2.1. Oilseed cakes

Three OC, sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC) were used. SFC was provided by Sorgal, S. A. and RSC and SBC were provided by IBEROL - *Sociedade Ibérica de Oleaginosas*, SARL. OC were dried at 65 °C for 24 hours and stored in hermetic plastic bags, kept in the dark at room temperature.

2.2.2. Microorganisms

Three fungi species were used. *Rhizopus oryzae* MUM 10.260 and *Aspergillus ibericus* MUM 03.113 were obtained from *Micoteca* of University of Minho, Braga, Portugal. *Aspergillus niger* CECT 2915 was obtained from CECT (Colección Española de Cultivos Tipo, Valencia, Spain). Fungi were cultivated in potato dextrose agar (PDA) plates and stored at 4 °C.

2.2.3. Characterization of unfermented and fermented solid substrates

OC were characterized regarding their physical-chemical properties. Initial and post fermentation characterization of OC was performed to monitor the changes due to the fermentative process. Moisture was calculated by drying the substrates in an oven at 105 °C during 24 h until constant weight. Ashes were determined by high temperature treatment at 575 °C for 2 h in a muffle. Nitrogen content was analyzed by the Kjeldahl method from which crude protein was estimated using a defined factor of 6.25. Carbon was analyzed following the method described by Leite et al.⁷ Total lipids were extracted from 0.4 g of sample using a mixture of chloroform: methanol (2:1; v/v) as extraction solvent. Briefly, the mixture was incubated at room temperature, with constant shaking at 0.5 g for 1 hour. After, lipids were filtered through a glass wool and evaporated to dryness. The lignocellulosic composition (cellulose, hemicellulose and lignin) of by-products was calculated by a quantitative acid hydrolysis as described by Leite et al.⁷ To estimate soluble protein, free sugars, total phenols and enzymatic activity an extraction with water (ratio solid: liquid of 1 g to 5 mL) was performed at 0.5 g at room temperature with mechanical agitation for 30 minutes. Following, the extract was filtered through a nylon net and the liquid fraction was centrifuged at 2264 g for 10 minutes at 4 °C. The enzymatic extract was recovered and stored at -20 °C until its analysis. Soluble protein was measured by the Bradford method and free sugars were analyzed by the 3,5-dinitrosalicylic acid (DNS) method. Total phenols were quantified using the Folin-Ciocalteu method (Commission Regulation (EEC) No.

2676/90). pH was measured on the moisturized solid using a probe and respective meter (Hanna Instruments edge® HI 2020-02).

2.2.4. Antioxidant activity

Radical scavenging activity was assessed by the DPPH method slightly modified as described by Dulf et al.,¹⁶ with adaptation to microplate reader. Briefly, 200 μ L of diluted sample extracts were mixture with 100 μ L of a methanolic solution of DPPH (0.5 mM). The mixtures were kept in the dark at room temperature for 30 min and then absorbance was measured at 517 nm. The free radical scavenging activity of extracts was expressed as micromoles of Trolox equivalents per gram of dry substrate (μ mol g^{-1}).

Iron chelating was evaluated by ICA method, performed as described by Oliveira et al.¹⁷ Extract's chelation ability was expressed as EDTA equivalents (nmol g^{-1}).

Superoxide radical scavenging activity was determined using the PMS-NADH non-enzymatic assay.¹⁷ The scavenging activity of the extracts was expressed as micromoles of ascorbic acid equivalents per gram of dry substrate (μ mol g^{-1}).

Ferric reducing ability of plasma (FRAP) was performed according to Fernandes et al.¹⁸ The reducing ability of extracts was expressed as micromoles of ferrous equivalents per gram of dry substrate (μ mol g^{-1}).

2.2.5. Hydration properties

Water holding capacity (WHC) was analyzed as according to Raghavendra et al.¹⁹ WHC was quantified by hydrating 1 g of dry sample with 30 mL of distilled water. The mixture was kept at room temperature for 18 h with stirring. The suspension was filtered, and the hydrated solid was dried at 105 °C for 2 h to obtain the residual dry weight. Values were expressed as gram of water per gram of dry solid.

2.2.6. Solid-state fermentation (SSF) of OC

SFC, RSC and SBC were used as substrate in SSF experiments to evaluate the effect of three fungi species, *R. oryzae*, *A. ibericus* and *A. niger*. SSF was carried out in 500 mL Erlenmeyer flasks with 10 g of dry substrate. Moisture was adjusted to 750 g Kg^{-1} (wet basis) with distilled water. Erlenmeyer flasks with the substrates were sterilized at 121 °C for 15 minutes. For inoculation, the fungi grown on PDA for 7 days were suspended in a sterile solution (1 g L^{-1} peptone and 0.1 g L^{-1} Tween 80) and adjusted to

a spore concentration of 10^6 spores mL^{-1} . Each substrate was inoculated with 2 mL spore suspension and incubated at 25 °C for 7 days. A control experiment was performed at the same SSF conditions but without inoculation. Each experiment was performed in duplicate.

2.2.7. Enzymatic activities

Cellulase (endo-1,4- β -glucanase) was determined using carboxymethylcellulose (CMC) as substrate. 250 μL of 20 g L^{-1} cellulase substrate in 0.1 M sodium acetate buffer, pH 4.8 was mixed with the same volume of enzyme containing sample. After enzymatic hydrolysis, reducing sugars were quantified by the DNS method. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose reducing sugar equivalents from CMC in 1 min at 50 °C and pH 4.8. The values of cellulase activity were expressed in units per gram of dry substrate (U g^{-1}).

The procedure to determine xylanase activity was the same as described for cellulase activity but the substrate solution used was xylan from beechwood g L^{-1} and the reaction time was 15 min instead of 30 min. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of xylose reducing sugar equivalents from beechwood xylan in 1 min at 50 °C and pH 4.8. The values of xylanase activity were expressed in units per gram of dry substrate (U g^{-1}).

β -glucosidase activity was determined using *p*-nitrophenyl- β -D-glucopyranoside (PNG) as substrate. In test tubes, 100 μL of 4mM PNG was mixed with 100 μL of sample in citrate buffer 0.05 N pH 4.8. The tubes were placed on a bath at 50 °C for 15 min. After 15 min, the tubes were transferred to an ice bath to cool down and then it was added 600 μL of Na_2CO_3 (1M) and 1.7 mL of distilled water. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol in 1 minute at 50 °C and pH 4.8. The values of β -glucosidase activity were expressed in units per gram of dry substrate (U g^{-1}).

Protease activity was quantified using azo casein (5 g L^{-1}) as substrate, in sodium acetate 50 mM, pH 5. 500 μL of sample was mixed with the same volume of substrate solution and incubated at 37 °C for 40 minutes. Afterwards, 1 mL of trichloroacetic acid 100 g L^{-1} was added to stop the reaction. Samples were centrifuged for 15 minutes at 600 *g*. Supernatant was recovered and after the addition of 1 mL of 5 N potassium hydroxide, the absorbance was read at 428 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of azopeptides in 1 minute at 37 °C and pH 5. Values of protease activity was expressed in units per gram of dry substrate (U g^{-1}).

2.2.8. Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) and post hoc Tukey's HSD test to discriminate means ($P < 0.05$). Principal Component Analysis (PCA) were carried out using Statgraphics Plus 5.1 (Manusgistics, Inc., Rockville, MD).

2.3 Results and discussion

2.3.1. Characterization of oilseed cakes

OC were physically and chemically characterized to assess their potential as substrate for SSF. **Table 2.1** shows the initial composition of OC.

OC used in this study are natural sources of protein, corresponding to 40–50% of dry matter. Nitrogen (N) and carbon (C) content are important parameters to take into account when assessing if a substrate is suitable to be applied in SSF. N stimulates fungal conidiation, while C represents the energy source to support microbial growth and metabolism. However, it was proven that the ratio of both contents (C/N) have higher significant effect in fungal growth and sporulation than C sources alone. It is estimated that an optimal C/N ratio is between 10-20, depending on the fungus strain.²⁰ SFC, RSC and SBC are rich in N and presented a suitable C/N ratio to be used as substrate in SSF studies without the need for external N supplementation. RSC and SBC C/N ratio were within the average values reported in the literature.^{21–23} SFC has a lower C/N ratio, and this is attributable to a higher N content. It is known that soil composition and fertility, location, rainfall, or temperature can influence the productivity and composition of crops. OC obtained after mechanical press contain small amounts of residual oil or fat, referred to as lipids. The OC treatments after oil extraction have a significant impact in the final OC composition. The amount of lipids in the present OC accounted for approximately 2% of dry matter. Comparing to literature, differences on OC composition may be related with genotypic and phenotypic variations between cultivars, conditions in which the cultures were grown or even the use of total or dehulled oilseeds.⁴ Additionally, the method used to treat oilseeds during oil extraction and the use of extraction solvents can affect OC final composition. Also, other factors not inherent to oilseeds may affect final composition of oilseed cakes such as the expression of results to dry matter or moist contents and the assays used. These factors may explain the low amount of total lipids observed in RSC in comparison to literature.

Table 2.1. Composition of oilseed cakes (g Kg⁻¹) and chemical composition of oilseed cakes reported in literature.

Parameter	SFC		RSC		SBC	
	Experimental	Literature	Experimental	Literature	Experimental	Literature
Humidity	98.23 ± 0.91	52.00 – 101.00 ^{21,24,25}	152.64 ± 3.00	80.20 – 101.00 ^{21,25,26}	132.13 ± 1.56	93.00 – 99.20 ^{23,26,27}
Ash	83.80 ± 0.14	52.60 – 75.00 ^{21,24,25,28}	66.58 ± 2.23	43.00 – 70.0 ^{21,26,28–30}	62.58 ± 1.03	58.30 – 74.80 ^{23,26–29}
Protein	401.99 ± 5.88	236.00 – 525.00 ^{21,24,25,28,29}	398.29 ± 20.86	338.00 – 403.00 ^{21,25,28,30}	504.11 ± 14.26	489.00 – 493.00 ^{26,28}
Nitrogen	69.50 ± 5.05	47.00 ²¹	76.44 ± 7.02	75.00 ²¹	91.07 ± 3.02	82.10 ²³
Carbon	466.62 ± 2.34	498.00 ²¹	505.88 ± 8.06	502.00 ²¹	473.49 ± 4.94	448.00 ²³
C/N ratio	6.71	9.8 – 10.60 ^{21,22}	6.62	6.69 – 8.40 ^{21,22}	5.20	5.46 ²³
Lipids	23.44 ± 3.91	27.50 – 303.00 ^{21,24}	20.42 ± 2.80	118.00 – 167.00 ^{21,30}	17.16 ± 1.62	21.00 ²⁷
Water Holding capacity	359 ± 35		193 ± 5		285 ± 30	500 ²¹
pH ^a	5.33		5.50		5.22	
Klason lignin	82.54 ± 0.51	77.20 – 133.00 ^{21,32,33}	77.42 ± 16.97	64.00 – 90.00 ^{21,29,33}	22.01 ± 1.35	10.00 – 28.00 ^{23,29}
Cellulose	142.79 ± 2.43	123.00 – 251.00 ^{21,32,33}	154.69 ± 16.35	59.00 – 81.80 ^{21,33}	161.54 ± 2.71	215.80 ²³
Hemicellulose	111.43 ± 6.68	44.20 – 154.00 ^{21,32}	136.27 ± 14.33	20.40 ^{21,33}	155.47 ± 6.53	525.10 ²³
NDF	336.76	216.00 – 453.00 ^{29,34}	368.38	200.00 – 299.00 ^{26,29}	339.02	111.00 – 158.90 ^{26,29}
ADF	225.33	145.00 – 372.00 ^{29,34}	232.11	141.00 ²⁹	183.55	78 ²⁹
Soluble protein ^b	7.92 ± 0.39		11.89 ± 0.66		8.41 ± 0.18	
Free sugars ^b	7.68 ± 0.06		9.70 ± 0.48		7.27 ± 0.32	
Total phenolic compounds ^b	11.68 ± 0.17		4.77 ± 0.16		3.10 ± 0.16	

SFC, sunflower cake; RSC, rapeseed cake; SBC, soybean cake; NDF, neutral detergent fiber; ADF, acid detergent fiber

^a Measured on the solid after aqueous extraction

^b Quantified in aqueous extracts

Cellulose and hemicellulose content were similar in the three OC while, SFC and RSC presented a higher concentration of lignin. Cellulose and hemicellulose fractions can act as inductors for the production of lignocellulolytic enzymes such as cellulases and xylanases, respectively.⁷ These enzymes are responsible for the degradation of cellulose and hemicellulose respectively, increasing OC digestibility in livestock. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) are good indicators of feed quality once they are correlated with digestibility by livestock animals. They are good indicators of dietary energy and intake, with focus for ruminant rations. All three OC presented similar values of NDF and ADF and were within the range of the values reported by literature with the exception of SBC that present slightly higher values. All the OC retained water that is critical in SSF. Water holding capacity (WHC) represents the water absorbed by the substrate fibers under limiting conditions and that can be used by the fungi to support its growth. Substrates used in SSF process usually have low water content. However, substrates with high WHC are normally selected for being used in SSF once the moisture can be easily modified according to the process needs. Ngoc et al.³¹ reported values of WHC for different fiber-rich plant sources and agro-industry by-products ranging from 2.5 and 8.0 (kg kg⁻¹ of dry matter). WHC is related with the content of lignocellulosic fractions. SBC had a lower WHC

compared to the reported in literature and this may be explained by the low concentrations of hemicellulose and cellulose. All cakes in this study had a similar pH value, around 5, which is found to be within the optimal range for filamentous fungal growth (3.8 to 6.0). The three OC contain small fractions of free extractable compounds such as phenolics, sugars and proteins. In the present work, these fractions were obtained through an aqueous extraction.

2.3.2. Antioxidant potential of oilseed cakes

Phenolic compounds are amongst the secondary metabolites produced by plants. **Figure 2.1.A** represents the concentrations of TPC, obtained after aqueous extraction, of each OC. SFC is the by-product with higher content of TPC. Its concentration is 1.94-fold higher than RSC and approximately 2.73-fold higher than SBC. It is known that OC contain high fractions of phenolic compounds. However, the presence of some of these compounds can hinder their use as animal feed as they can negatively affect protein digestibility from OC due to complex protein-phenolic compound interactions. On the other hand, other phenolics may present relevant bioactivities, since they may provide health benefits due to antioxidant properties, induction or inhibition of enzymes and gene expression.³⁵ **Figures 2.1.B-E** depicts the results of antioxidant analysis of sample extracts. Regarding capacity to scavenge the DPPH radical (**Fig. 2.1.B**), the maximum value was obtained in SFC extract, that was 2 and 6-fold higher than the values obtained with RSC and SBC respectively. The scavenging of free radicals of each extract is in agreement with the concentration of TPC. **Figure 2.1.C** represent the ability to chelate iron of the extracts. This metal catalyzes oxidation process resulting in a decrease of hydroxyl radical generation through Fenton reactions. Aqueous extracts of SFC had the highest capacity to chelate iron, and the values obtained with SFC, and SBC did not present statistically significant differences ($P < 0.05$). The low capacity to chelate iron of RSC extracts may be explained by a different nature of phenolics present in this extract. Iron is an element that can undergo cyclic oxidation and reduction and is involved in the Fenton reaction leading to the production of ROS that are capable of oxidizing a range of organic substrates.

Superoxide anion is formed within the mitochondria, peroxisomes and endoplasmic reticulum.³⁶ This anion is one of the three primary species of ROS, alongside hydrogen peroxide and hydroxyl radical. They can react with organic substrates leading to the production of intermediate species able to produce more ROS.³⁷ From **Figure 2.1.D** is possible to observe that SFC extracts presented the highest superoxide anion scavenging capacity among the three OC extracts. SFC scavenging capacity

was 4.8-fold higher than RSC and SBC, with no statistically significant differences ($P < 0.05$) between the results among these two OC.

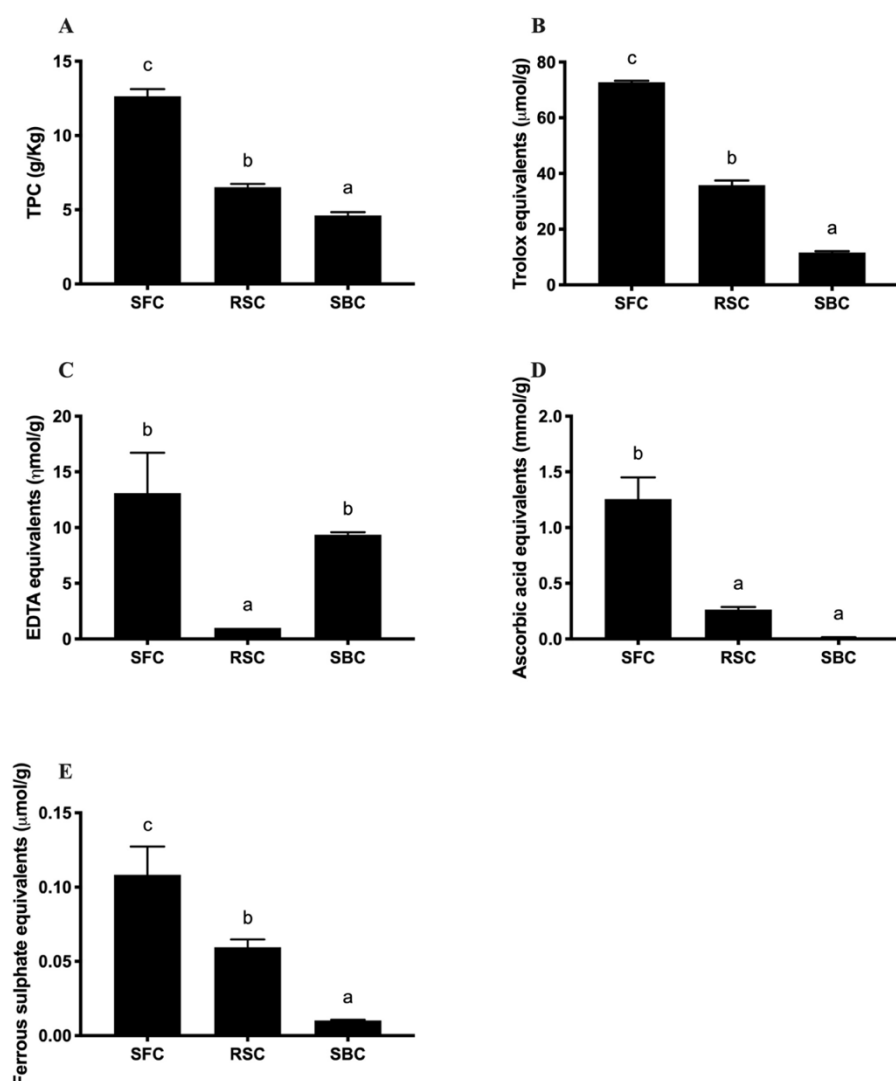


Figure 2.1. Total phenolic content (TPC) and antioxidant potential of aqueous extracts of non-fermented oilseed cakes. **(A)** TPC; **(B)** DPPH radical scavenging activity; **(C)** iron chelation ability; **(D)** superoxide radical scavenging activity; **(E)** reducing ability. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters are not statistically significant different (Tukey test; $P < 0.05$).

Figure 2.1.E show the results of FRAP assay of OC extracts. This method is based on the antioxidant strength in reducing the ferrous tripyridyltriazine (Fe^{III} -TPTZ) complex to its stable form (Fe^{II} -TPTZ). Extracts that exhibit antioxidant capacity in this assay, normally are electron donors as they reduce the Fe^{III} -TPTZ to its stable form interrupting the oxidation chain.³⁸ Results follow a similar trend as the ones obtained through the DPPH assay. Statistically significant differences ($P < 0.05$) were observed between

the three extracts. SFC extracts have the highest reducing power, that is 1.86-fold higher than the obtained value in RSC. SBC extract showed the lowest reducing power being 11-fold lower than SFC.

In general, it may be concluded that SFC is the OC with more potential for antioxidant compounds extraction by an eco-friendly solvent (water). Extracts of SFC are rich in antioxidant phenolic compounds. Mohdaly and Ramadan³⁹ reported DPPH scavenging potentials between 46 and 71% of different *Brassica carinata* genotypes. From the 20 *Brassica carinata* genotypes studied, only two achieved a scavenging potential near SFC. Comparing to agro-industrial wastes from winery, brewery and olive oil industry namely vine shoot trimmings, exhausted grape marc, grape stalks, crude and exhausted olive pomace and brewers spent grain, SFC antioxidant potential is considerably high, achieving scavenging potential values between 1.44 and 14.4-fold higher than the referred residues.¹⁰

2.3.3. Production of enzymes by SSF

OC were used as substrate in SSF with three fungi species. **Figure 2.2.** show the activity of cellulases **(A)**, xylanases **(B)**, β -glucosidases **(C)** and proteases **(D)** obtained after SSF. Cellulases maximum activity, was achieved with *A. niger*, using SBC as substrate. *A. niger* was in general the best producer of cellulases in the three OC at the SSF conditions used, however no statistically significant differences ($P < 0.05$) were found between cellulases produced by *A. niger* and *A. ibericus* using SFC as substrate. Maximum xylanases activity was obtained using RSC as substrate fermented by *A. niger* and this enzyme production was 66%, 85% and 69% (SFC, RSC and SBC, respectively) higher than the obtained with *A. ibericus*, that was the second largest producer.

On the other hand, maximum activity of β -glucosidases was achieved with *R. oryzae* and *A. ibericus* using RSC as substrate, being similar ($P < 0.05$) amongst them. Regarding SFC and SBC, no statistically significant differences ($P < 0.05$) were observed between enzymes produced by each fungus.

The use of SFC and canola (a cultivar of rapeseed) for the production of cellulases and xylanases, using different *Aspergillus* species, was evaluated by de Castro et al.²² Maximum enzyme activity for these substrates was achieved using *A. awamori* IOC-3915. They reported activity values of 9.7 U g⁻¹ and 6.1 U g⁻¹ for cellulases and 413.5 U g⁻¹ and 571.4 U g⁻¹ for xylanases activity, respectively for each substrate. *A. niger* IOC-4003 produced cellulases activity of 1.1 U g⁻¹ and 0.2 U g⁻¹ and xylanases activity of 40.6 U g⁻¹ and 13.7 U g⁻¹ using SFC and canola cake as substrate, respectively. The use of soybean meal as substrate for a multienzyme production was evaluated by Vitcosque et al.⁴⁰ They observed a maximum xylanases production of 47.7 U g⁻¹, using *A. niger*. Despite the production of β -glucosidases using OC as

substrate for SSF has not been yet extensively explored, studies using other lignocellulosic materials to produce this enzyme have been performed. Leite et al.,¹⁰ reported a maximum activity of 93.66 U g⁻¹ of β -glucosidases for *A. niger* CECT2088 using brewer's spent grain as substrate. Filipe et al.,⁴¹ optimized the production of β -glucosidases using olive mills and winery wastes as SSF substrate, achieving maximum enzyme activity of 25.5 U g⁻¹ and 17.9 U g⁻¹ for *A. ibericus* MUM 03.49 and *A. niger* CECT 2915, respectively. Ezeilo et al.,⁴² reported that *R. oryzae* presented maximum β -glucosidases activity of 145.47 U g⁻¹ after optimization of SSF parameters, using raw oil palm frond leaves as substrate.

Apart from lignocellulolytic enzymes produced by filamentous fungi during SSF, several other enzymes can be produced using this process. Proteases are among the wide spectrum of enzymes that can be produced by SSF.⁶

Maximum protease activity was achieved with SFC and *A. niger* however, no statistically significant differences ($P < 0.05$) were found between those values and the ones obtained with the other fungi in SFC. SSF of RSC and SBC led to lower production of protease with all the fungi than the obtained with SFC but *A. niger* was the best producer ($P < 0.05$) in these OC. *A. niger* is one of the most important sources of fungal proteases once, its genome sequencing by Pel et al.⁴³ revealed 198 proteins involved in proteolytic degradation process. de Castro et al.²² reported proteases production of 0.8 and 0.5 U g⁻¹ using SFC and canola cake as substrates for SSF. In the same work it was reported maximum proteases production of 9.9 and 12.6 U g⁻¹, using SFC and canola cake as substrate for SSF when fermented with *A. awamori* IOC-3915 and *A. sulphureus* respectively. Mukhtar⁴⁴ achieved maximum protease activity of 5.2 U g⁻¹, 3.3 U g⁻¹ and 4.8 U g⁻¹ of dry substrate, for sunflower meal, rapeseed meal and soybean meal, respectively, using *A. niger* IGH₉.

Overall, *A. niger* was the fungus that produced higher activities of enzymes mainly using SFC and RSC as substrate. On the other hand, SBC favored the production of cellulase.

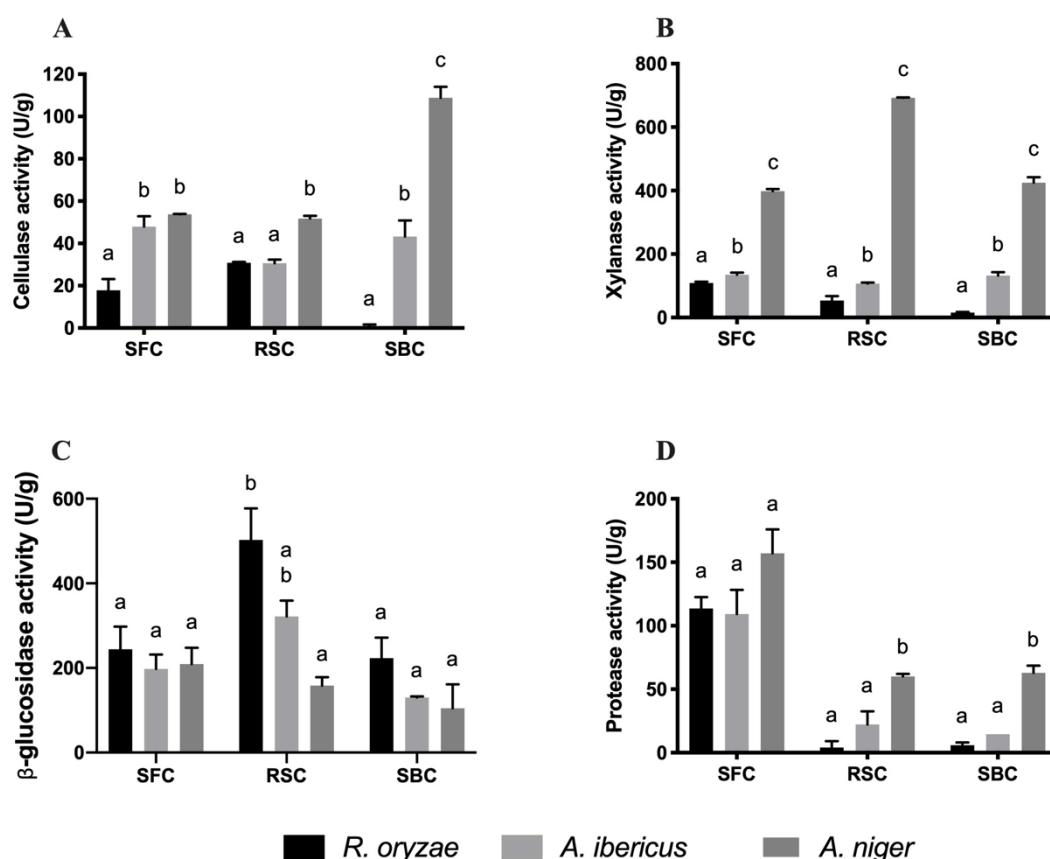


Figure 2.2. Cellulases (**A**), xylanases (**B**), β -glucosidases (**C**) and proteases (**D**) production during SSF. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters are not statistically significant different (Tukey test; $P < 0.05$). Statistical analysis is relative to each oilseed cake.

Enzymatic activity values achieved during this work easily outpace the ones reported by other authors. Cellulase maximum activity of 109 U g^{-1} exceed the maximum production of 84.2 U g^{-1} reported by Filipe et al.⁴¹ by 1.3-fold. Regarding xylanase, an increased difference of 100 U g^{-1} was obtained comparatively to the maximum of 598 U g^{-1} reported by de Castro et al.²² In relation to β -glucosidase production an increase of approximately 71% was observed from the maximum of 145 U g^{-1} reported by Ezeilo et al.⁴² and the maximum obtained in this work of nearly 503 U g^{-1} . Additionally, protease maximum production of 157 U g^{-1} was around 30-fold higher than the maximum of 5.2 U g^{-1} , reported by Mukhtar⁴⁴.

2.3.4. Antioxidant capacity of extracts of fermented oil cakes

TPC and antioxidant potential of aqueous extracts of fermented OC were evaluated and compared with unfermented sterilized OC. According to the results in **Figure 2.3.A**, SSF had a positive effect, comparing to controls, on the increment of TPC concentration in the extracts obtained with all the fungi in RSC and SBC, and with *R. oryzae* in SFC, where the action of the other fungi did not affect the TPC liberation. For SSF with *R. oryzae* the increase of TPC in the extracts compared to controls was approximately of 2.6-fold in SFC, 5.6-fold in RSC and 10-fold in SBC. These results indicate the importance of SSF for TPC extraction of OC that are more resistant to direct extraction process with eco-friendly solvents.

TPC increase during SSF may be related with the action of lignocellulolytic enzymes. Insoluble phenolic compounds can be found connected to structures of the vegetable cell wall such as arabinose or galactose residues of hemicellulose components and also to lignin, which is a network of phenolic compounds.⁴⁵ Filamentous fungi possess two extracellular enzymatic systems, an hydrolytic one that produces hydrolases, able to degrade polysaccharides and an oxidative system, responsible for the degradation of lignin and opening of phenyl rings, increasing free phenolics.⁴⁶ The decomposition of linkages between lignin, cellulose or hemicellulose increases the release of phenolic compounds.

Highest concentration of TPC were observed in fermentation performed with *R. oryzae* which, also reported the maximum β -glucosidase activity. Sheih et al.⁴⁷ observed that β -glucosidase, alongside α -amylase were responsible for the mobilization of phenolics during *A. niger* M46 fermentation.

Figures 2.3.B-E show the results obtained of four different antioxidant assays performed with OC fermented extracts to assess their antioxidant potential. Regarding the ability to scavenge free radicals (**Fig. 2.3.B**), maximum antioxidant potential was obtained in aqueous extracts of RSC fermented with *A. niger*, that was the fungi with which highest values of antioxidant potential were achieved in fermentations performed with all OC. *A. niger* was able to increase antioxidant potential of extracts in approximately 2, 4.6 and 14.3-fold for SFC, RSC and SBC, respectively. Surprisingly, *R. oryzae* fermented extracts achieved lower values of antioxidant potential than *A. niger*. These results indicate that the phenolic compounds that are liberated by the action of these fungi species are different and may be related with the action of enzymes most produced by each species, as it was found in this work. In fact, the direct correlation of TPC and antioxidant capacity may be difficult to establish as it found by Terpinc et al.⁴⁸ that studied the correlation between TPC and antioxidant potential of different oil cake extracts. They observed a negative correlation between these two parameters. Additionally, the authors suggested that samples with similar concentrations of TPC vary significantly in their antioxidant activity.

The same was observed in this work between fermented extracts of *A. ibericus* and *A. niger*. This fact may be explained by the synergistic and antagonistic interactions between the antioxidants. The action of lignocellulolytic enzymes such as cellulases and xylanases can also lead to antioxidant potential differences once the hydrolysis of some phenolic conjugates, present in the lignocellulosic matrix of plant cell walls, can release free phenolic compounds and low molecular weight molecules with higher antioxidant potential.

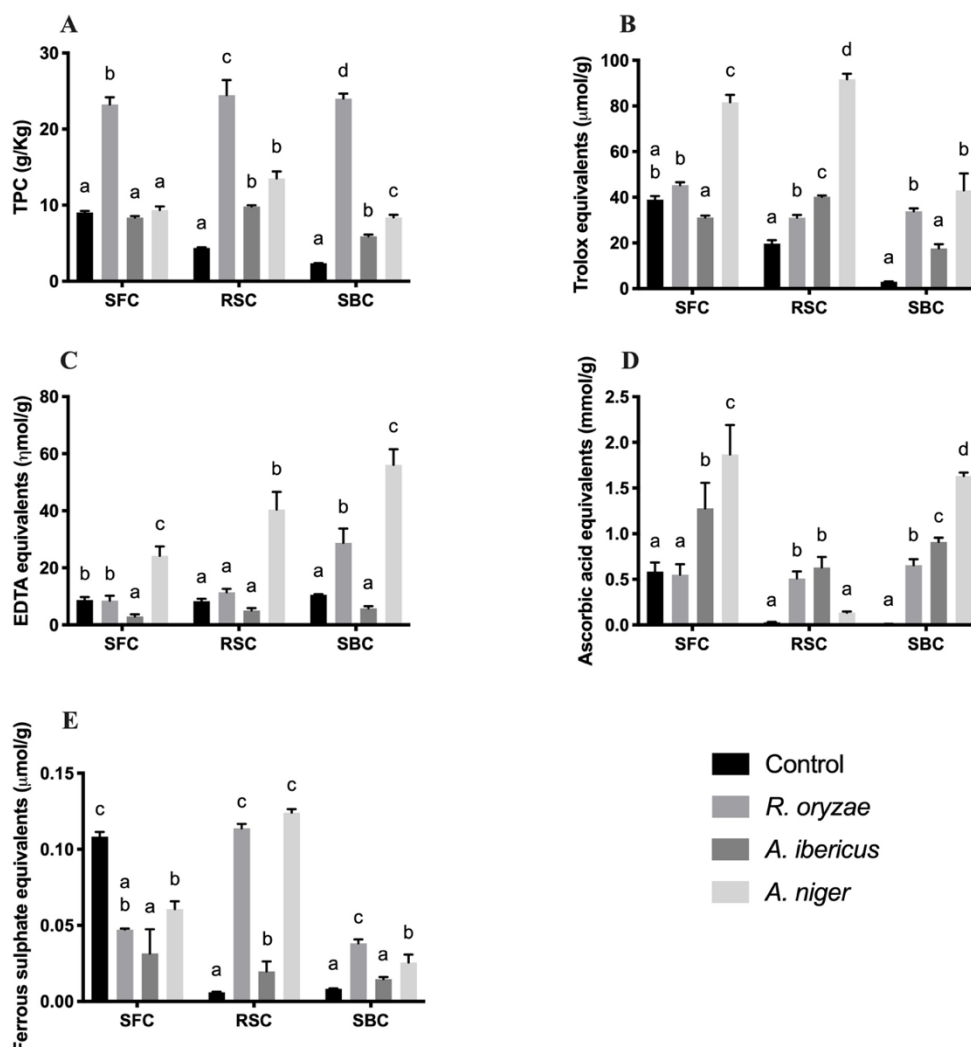


Figure 2.3. Total phenolic content (TPC) and antioxidant potential of aqueous extracts of controls (oilseed cake sterilized) and fermented oilseed cakes. **(A)** TPC; **(B)** DPPH radical scavenging activity; **(C)** iron chelation ability; **(D)** superoxide radical scavenging activity; **(E)** reducing ability. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters are not statistically significant different (Tukey test; $P < 0.05$). Statistical analysis is relative to each oilseed cake.

The highest antioxidant potential of extracts from fermented OC with *A. niger* was also observed in the results of the ability to chelate iron (**Fig. 2.3.C**) where the maximum capacity was observed in fermented extracts of SBC. Generally, SSF increased capacity to chelate iron of the extracts compared to the control, with the exception of SFC when fermented with *A. ibericus*.

With respect to the superoxide scavenging potential (**Fig. 2.3.D**), fermented extracts showed a significant increase of antioxidant potential regarding the controls. Maximum scavenging potential was observed in *A. niger* extracts with SFC. *A. niger* was responsible for the highest increases in extracts scavenging potential in SFC and SBC. A 3.22-fold increase was observed in SFC extracts while in SBC the increment was near 233-fold. Concerning RSC, *A. ibericus* lead to a 21.7-fold increase of scavenging potential.

As shown in **Figure 2.3.E**, SFC fermented extracts reducing power decreased around 57% when compared to control. SFC presented a relatively higher reducing power when compared to RSC and SBC. In this case, the action of the microorganisms may lead to a decrease of electron donor species due to their consumption or lead to the aggregation of species causing a decrease in the reducing power capacity. On the other hand, regarding RSC and SBC fermented extracts it was observed a clear increase of reducing power ability. Maximum reducing power was observed in fermented extracts of RSC with *R. oryzae* and *A. niger*, showing no statistically significant differences ($P < 0.05$). This is a 20-fold increase while on SBC extracts it was observed a 4.75-fold increase.

SSF has been widely used to improve the nutritional properties of agricultural products and to obtain bioactive compounds. In this work, SSF was able to increase the antioxidant capacity of extracts as previously described by other authors for other substrates. Zhai et al.⁴⁹ reported an increase of 89%, 66.1%, 86.5% and 72.9% regarding DPPH radical scavenging ability, ferrous ion chelating ability, superoxide anion radical scavenging ability and reducing power, respectively, of ethanolic extracts of corn fermented with *Agaricus brasiliensis* SH26. The same author reported an increase of 99.3%, 87.4%, 85% and 91.4% for the same four antioxidant indexes of ethanolic extracts of corn fermented with *A. bisporus* 2796. Xu et al.⁵⁰ tested the effect of SSF on the antioxidant properties of ten cereal grains. This author reported an increase of antioxidant potential of all cereal grains regarding their ability to scavenge the DPPH radical, reducing power ability, ferrous ion chelating activity and superoxide anion radical scavenging capacity of ethanolic extracts. Kang et al.⁵¹ reported the increase of buckwheat antioxidant potential regarding the DPPH and superoxide scavenging abilities, reducing power and ferrous ion chelating activity after SSF with *A. blazei* and *A. bisporus*.

2.3.5. Nutritional parameters of fermented oil cakes

Relevant physico-chemical parameters to assess nutritional quality of fermented OC were evaluated on fermented and extracted OC. **Figure 2.4.A** shows that in SSF experiments using *A. niger*, no loss of crude protein was found in the fermented OC.

However, with *R. oryzae* and *A. ibericus*, there was a decrease in crude protein during the SSF process. SSF has been used over the past decades to improve the nutritional value of agricultural wastes, namely protein content. In a previous work, SSF of brewer's spent grain, exhausted olive pomace, exhausted grape pomace and vine-shoots trimming was successfully applied for the production of single cell protein, using different *Aspergillus* strains.⁵² Canedo et al.⁵³ was able to increase crude protein of brewer's spent grain by 2-fold, using the genus *Rhizopus* in SSF.

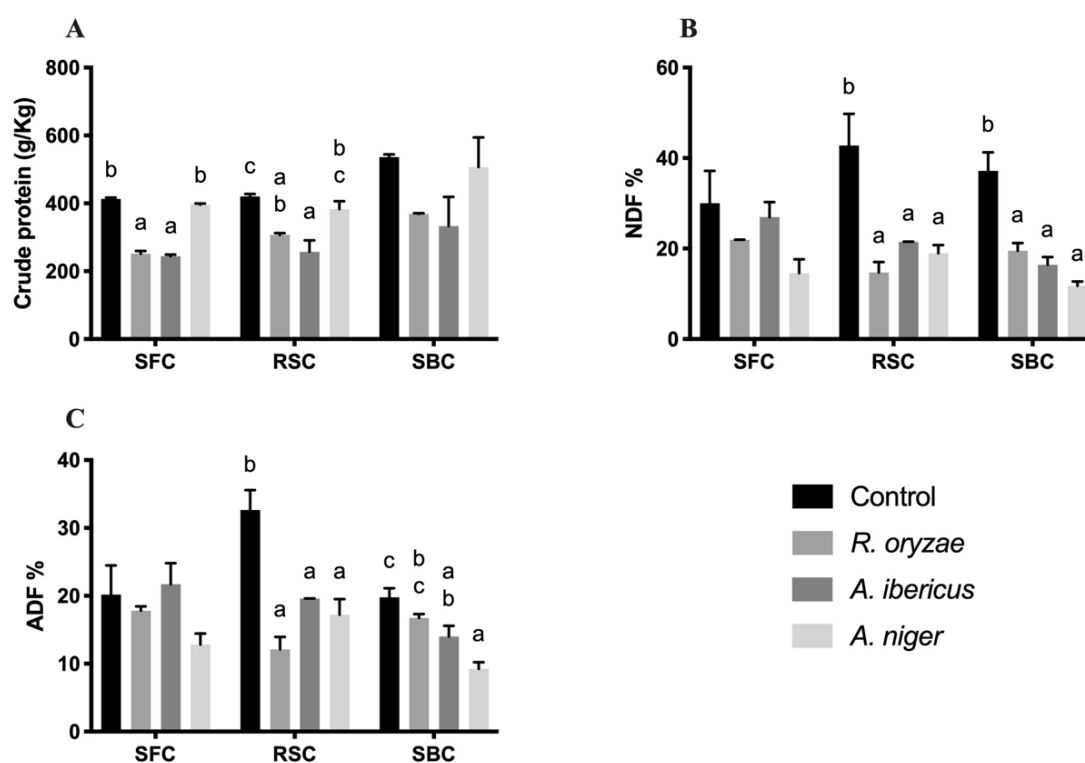


Figure 2.4. Crude protein (A), neutral detergent fiber (NDF) (B) and acid detergent fiber (ADF) (C) of controls (oilseed cake sterilized) and fermented oilseed cakes. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters are not statistically significant different (Tukey test; $P < 0.05$). Statistical analysis is relative to each oilseed cake.

However, in this study, the decrease in crude protein content using *R. oryzae* and *A. ibericus* could indicate that these fungi can use the available N to support their growth and differentiation, as there was not any external N supplementation. During SSF, lignocellulosic fractions are affected by the activity

of microorganisms. Filamentous fungi degrade the lignocellulosic matrix into small soluble sugars that can be further consumed by the microorganisms. Compositional changes regarding dietary fibers are quite important during bioconversion studies, such as SSF, once these fractions can affect OC digestibility by animals. NDF and ADF fractions were thus evaluated during this work (**Fig. 2.4.B** and **2.4.C**, respectively). Decrease of these fractions was observed in all fermented OC with the exception of SFC for which NDF and ADF content values of fermented OC were not statistically significant different ($P < 0.05$) from the non-fermented ones. Decreases in cell wall components of 53%, 67% and 69% were observed in SFC, RSC and SBC, respectively, regarding NDF composition. Additionally, ADF decreases of 37%, 63% and 53% were observed for SFC, RSC and SBC respectively. These results may be explained by the action of fungal enzymes produced during SSF that breakdown lignocellulosic bounds between lignin, cellulose and more particularly with hemicellulose. The fiber reduction is an important result of nutritional upgrade of OC for their application as animal feed. The use of filamentous fungi to increase the nutritional value of OC to be used in animal feed does not entail any obstacle and is a viable technique due to the nature of the species used. The fungi used during this study are recognized as GRAS by the US Food and Drug Administration (FDA). *Aspergillus niger* is one of the most used species in SSF. *A. ibericus* do not produce any relevant toxins and *Rhizopus oryzae* has been used in SSF studies using agro-food residues for the production of extracellular enzymes.^{6,52,54}

2.3.6. PCA analysis

A principal component analysis (PCA) was performed in order to assess the relationship between substrates, fungi, total phenolic compounds, antioxidant capacity, enzymes produced, crude and soluble protein and lignocellulosic fractions (NDF and ADF). PCA allowed to obtain a reduced number of linear combinations of the 13 variables studied to explain data variability. With this analysis, four main components were identified and explain 84.59% of the variability of original data. The first two principal components, PC1 and PC2 are shown in **Fig. 2.5.**, and they accounted for 61% of the variability (42% and 19% respectively). PC1 was positively characterized by high enzymatic activity (xylanase, cellulase, protease and β -glucosidase), total phenolic compounds (TPC) and antioxidant potential. On the other hand, PC1 was negatively correlated with neutral detergent fiber (NDF) and acid detergent fiber (ADF). As seen in **Fig. 2.5.**, substrates with higher concentrations of lignocellulosic fractions lead to a higher production of xylanase and cellulase. The deconstruction of lignocellulosic materials due to enzymatic activity is also positively correlated with an increased antioxidant potential.

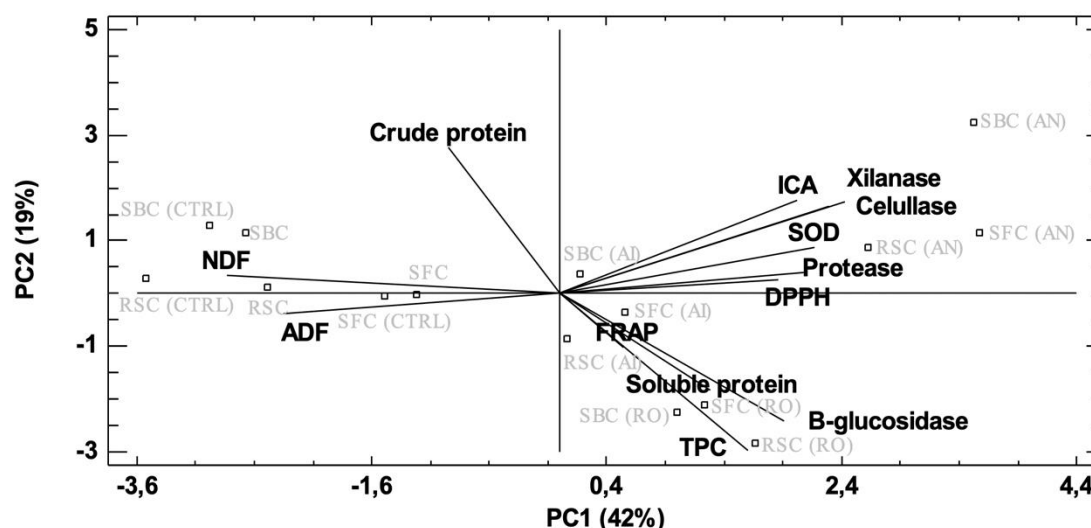


Figure 2.5. Principal component analysis. Biplot representation of variables and SSF experiments performed.

CTRL, control; RO, *R. oryzae*; AI, *A. ibericus*; AN, *A. niger*.

For PC2, crude protein and NDF were correlated positively while TPC, β -glucosidase and soluble protein were correlated negatively. It is possible to observe the effect of crude protein in protein solubilization and a relationship between β -glucosidase activity and increase of TPC. Despite having a similar composition, PCA grouped OC and controls with respect to their fiber content according to the following order: SBC > RSC > SFC. Additionally, with this analysis it was possible to correlate fungus strain with their main bioactivity. *R. oryzae* was characterized by a high production of β -glucosidase and TPC alongside protein solubilization. *A. ibericus* was lightly correlated with antioxidant potential and enzymes production. *A. niger* was highly correlated with enzymes production (cellulase, xylanase and protease) and antioxidant potential (ICA, SOD, DPPH).

2.4 Conclusions

Degradation of lignocellulosic fractions from OC by fungi extracellular enzymes was demonstrated and this can favor nutrient digestibility of OC by farmed animals. Also, this degradation led to the release of TPC with potential antioxidant capacity. This study showed the potential of OC to be used in SSF to obtain new fermented and upgraded residues as well as enzymatic rich extracts with antioxidant capacity, that also have potential to be applied in the food and feed industry. The use of biotechnological process for valorization of by-products without generating any additional waste is the key to a sustainable development.

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Chapter 3

Bioprocessing of oilseed cakes by fungi consortia: impact of enzymes produced on antioxidants release

This chapter is based on the following original research article:

Sousa, D.; Salgado, J. M.; Cambra-López, M.; Dias, A.; Belo, I. Bioprocessing of oilseed cakes by fungi consortia: impact of enzymes produced on antioxidants release. *Journal of Biotechnology* **2023**. <https://doi.org/10.1016/j.jbiotec.2023.01.008>."

Abstract

Oilseed cakes (OC) present high potential as feedstock for the biobased industry. Biotechnological processes allow OC valorization by the production of diverse value-added products and simultaneously altering OC structure, improving their nutritional value, and boosting OC utilization in animal feed. This work explored the use of fungi consortium of two different species as a bioprocessing approach to improve the nutritional quality of OC, obtain enzymes and antioxidants by solid-state fermentation (SSF) of sunflower cake (SFC) and rapeseed cake (RSC). *Rhizopus oryzae* and *Aspergillus ibericus* consortium led to the highest production of cellulase (135 U/g) and β -glucosidase (265 U/g) while maximum protease (228 U/g) was obtained with *A. niger* and *R. oryzae* consortium. Maximum xylanase production (886 U/g) was observed in SSF of RSC resulting in high hemicellulose reduction. The synergistic action of lignocellulosic enzymes resulted in fermented extracts with increased antioxidant potential with possible application as food additives against oxidative stress.

Keywords: co-cultures, filamentous fungi, antioxidants, enzymes, crude protein, fiber

3.1. Introduction

Lignocellulosic biomass is a widely bioavailable, natural, and promising material that has been exploited in many biotechnological processes. Biomass is obtained from atmospheric carbon dioxide (CO₂) and water, using sunlight as the energy source for the photosynthesis process.¹ Agricultural wastes, industrial by-products, woody materials, energy crops, and human waste are some of the potential sources of lignocellulosic biomass. Global lignocellulosic biomass production accounts for approximately 181.5 billion tons annually.² Although the composition of this resource may change according to its origin, they are mainly composed of carbohydrates such as cellulose (35 – 50 %) and hemicellulose (25 – 30 %), lignin (15 – 20 %), lipids, ashes, and soluble components including proteins, sugars and phenolic compounds.^{3,4}

The rapid and accelerated rate of global climate change implies a quick transition to a sustainable society that can reuse and valorize natural bioresources, such as lignocellulosic biomass. Biomass is generally complex and thus pre-treatments are essential to overcome their compound structure and obtain maximum profit from their components.

The vegetable oils industry is one of the main sources of lignocellulosic biomass. The impact of vegetable oils on human health is invaluable. They are rich in essential unsaturated fatty acids and various valuable bioactive compounds that contribute to the absorption of fat-soluble vitamins and ensure the normal function of the endocrine system.⁵ Additionally, the exploitation of vegetable oils for biodiesel production as a replacement for fossil fuels largely contributes to the increased production of oilseeds. It is expected that by 2029, 87% of world oilseeds production, excluding soybean, will be crushed.⁶ Solid by-products obtained after mechanical extraction of oil are referred to as cakes and are of lignocellulosic nature. The global production of these commodities has increased over the past years. Edible oil cakes are nutritionally valuable and can be used as a supplement in human diets, as an additive in bakery, infant food, or as multipurpose supplements.⁷ Non edible oil cakes are used as green manure due to the presence of toxic compounds.⁸ Application as animal feed, landfilling, or biofuel conversion (heat or electricity) are among the most conventional practices to capitalize oilseed cakes.⁹ However, the presence of toxic compounds and antinutritional factors can affect animals' and humans' well-being. Sustainable improvement of food chains is essential to overcome these issues resulting in value-added products.

Sunflower cake (SFC) and rapeseed cake (RSC) are two of the most generated by-products obtained from the vegetable oils industry. These by-products are characterized for their high protein content, lipids, carbohydrates, and minerals. SFC has a protein content ranging from 23-53% while crude fiber

may vary from 17-33%.^{7,10} RSC has a high protein content between 14-40% and 5-15% of crude fiber.⁷ Additionally, these OC contain phenolic compounds that act as antioxidants against oxidative stress.^{7,10} Total carbon accounts for approximately 50% (w/w) of these products ensuring they can be used as substrate in solid-state fermentation (SSF) as the carbon source for microorganism's growth and metabolites production.

SSF is defined as a fermentation process that occurs in the absence or near absence of free water to which is applied a natural or inert support used as substrate for the growth of microorganisms.¹¹ However, the solid substrate must contain enough moisture to support the growth and metabolism of microorganisms. In this type of fermentation, microorganisms grow in conditions close to their natural *habitat* and filamentous fungi are the ones that better adapt to this environment.

The use of SFC and RSC as substrate for SSF, as a biological approach of OC treatment, has the potential to increase the nutritive value of these products.¹⁰ Deconstruction of the lignocellulosic matrix through extracellular hydrolytic enzymes produced by fungi may favor the release of unextractable conjugated phenolics with potential antioxidant capacity. Phenolic acids, flavonoids, and lignans can be found in OC as free, esterified, or in condensed form. The action of these compounds can help reduce oxidative stress preventing the appearance of various types of cancer.⁷ Phenolic compounds are the main type of antioxidants found in SFC and RSC. Amongst them, catechin, epicatechin, *p*-coumaric, and chlorogenic acid are the main antioxidants found in SFC while gallic acid, caffeic acid, ferulic acid, quercetin, luteolin, and sinapic acid are the major antioxidants present in RSC.¹²⁻¹⁴ Phenolic compounds have redox properties which are related to the extinction of singlet and triplet oxygen, to decompose peroxides and absorb and neutralize free radicals.

Despite the commercial value of OC and its applications, through clean and low-cost bioprocess it is possible to increase their value and, in the same process, obtain value-added bioactive components. Through SSF it is possible to obtain commercially interesting enzymes with possible applications in industries such as textile, paper, or as a supplement in animal feed.¹⁵ Furthermore, valuable phenolic compounds with possible applications in the pharmaceutical, cosmetic, or food area as antioxidants, anti-inflammatory, chemoprotective or anti-carcinogenic agents can be obtained.¹⁶ Finally, SSF can contribute to obtaining an improved residue with increased nutritional properties such as reduced fiber content to be used in animal feed.

Understanding the behavior of isolated fungal strains in the biotechnological process is a step further for process optimization. SSF of OC using single filamentous fungi species has been previously described by Sousa et al.¹⁰ Those single cultures, under the same conditions, showed different profiles

of enzymatic activity and antioxidant potential. While *Rhizopus oryzae* proved to be the best producer of β -glucosidase leading to a higher concentration of total phenolic compounds (TPC) in fermented extracts, *Aspergillus niger* showed to be a good producer of cellulases and xylanases while having an important role in the obtainment of antioxidant-rich extracts and maintenance of crude protein. Microbial consortia can contribute to increasing process yields because of the high demand from industries, to obtain a more complete cocktail (enzymatic or antioxidant) or even to overcome individual limitations of monocultures. Fungi consortia occur ubiquitously in nature and the symbiotic relations between species allow them to suppress monoculture limitations. Synergistic interactions between fungal species may increase substrate use resulting in higher productivity, increased fungal species adaptability to the fermentative environment, and increased resistance to contamination by unwanted microorganisms.¹⁷ Additionally, nutritional limitations may be overcome by the interaction between compatible species. The use of fungal consortia can be a viable and interesting alternative to increase the catalytic potential of enzymatic cocktails. The successful obtainment of enzymatic rich extracts with increased activity over monocultures was reported by some authors using lignocellulosic residues such as cottonseed cake, sugarcane bagasse, and wheat bran.^{18–21} However, less exploited is the use of fungal consortia to enhance the extraction of phenolics with relevant antioxidant potential.

This study evaluates the use of fungi consortium for fermentation of OC under SSF conditions. The work aimed to evaluate the potential use of fungi co-cultures on SSF of SFC and RSC to produce lignocellulolytic enzymes and antioxidant compounds, as well as to modify the nutritional properties of OC to improve their applications in animal feed.

3.2. Materials and methods

3.2.1. Oilseed Cakes

Two OC from vegetable oils production industries were used: SFC and RSC. OC were obtained from industries operating in Portugal. SFC was provided by Sorgal, S.A. and RSC was provided by IBEROL – *Sociedade Ibérica de Oleaginosas*, SARL. OC were dried at 65 °C for 24 hours and kept in closed containers in the dark at room temperature. These OC were previously characterized,¹⁰ having both around 40% (w/w) of protein content, 8% of lignin, with slight differences in cellulose (14% to 16%), and hemicellulose of 11% and 14% for SFC and RSC, respectively.

3.2.2. Microorganisms

SFC and RSC were used as substrates in SSF experiments to assess the effect of fungi consortium using three fungi species, *R. oryzae*, *Aspergillus ibericus*, and *A. niger*. *R. oryzae* MUM 10.260 and *A. ibericus* MUM 03.113 were obtained from *Micoteca* of the University of Minho, Braga, Portugal. *A. niger* CECT 2915 was obtained from CECT (Colección Española de Cultivos Tipo, Valencia, Spain). Fungi were cultured in potato dextrose agar (PDA) plates and preserved at 4 °C.

3.2.3. Solid-state fermentation

SSF experiments were carried out using 500 mL Erlenmeyer flasks, containing 10 g of dry substrate with moisture adjusted to 75% (w/w, wet basis) with distilled water. Flasks were sterilized at 121 °C for 15 minutes. For inoculation, a sterile peptone solution (1 g L⁻¹ peptone and 0.1 g L⁻¹ Tween 80) was added into PDA slants to collect spores and the suspension concentration was adjusted to allow an initial fermentation inoculum of 2x10⁵ spores g⁻¹ (per mass of dry solid). Flasks were kept at 25 °C for 7 days. Each experiment was performed in duplicate.

3.2.4. Extraction of bioactive compounds

Antioxidant phenolic compounds and enzymes were recovered after SSF through liquid/solid extraction using 50 mL of distilled water. Mixtures were stirred for 30 min in an orbital incubator at 20 °C and afterward, were filtered through a fine-mesh net. The resulting liquid fraction was centrifuged at 2264 *g* for 10 min at 4 °C. The enzymatic extract was recovered and stored at -20 °C until analysis.

3.2.5. Total phenolic compounds and antioxidant activity

Total phenolic compounds (TPC) were determined using the Folin-Ciocalteu method (Commission Regulation (ECC) No. 2676/90) with some modifications.¹⁰ TPC was expressed as mg GA per g of dry matter.

Antioxidant activity accessed by the DPPH method was performed as described by Dulf et al.²² with some modifications.¹⁰ The free radical scavenging potential of extracts was expressed as micromoles of Trolox equivalents per gram of dry matter (μmol g⁻¹).

Antioxidant potential quantified by the iron chelating activity (ICA) was performed as described by Sousa et al.¹⁰ Extract's capacity to chelate ferrous ion was expressed as EDTA equivalents (nmol/g).

Extracts capacity to scavenge the superoxide radical was determined using the PMS-NADH non-enzymatic assay as described by Gangwar et al.²³ with some modifications.¹⁰ The scavenging potential of fermented extracts was expressed as micromoles of ascorbic acid equivalents per gram of dry matter ($\mu\text{mol g}^{-1}$).

The Ferric Reducing Antioxidant Power Assay (FRAP) was performed according to Benzie & Strain,²⁴ with some modifications to adapt the assay for microplate.¹⁰ The reduction potential of fermented extracts was expressed as ferrous sulfate equivalents ($\mu\text{mol/g}$). All assays were performed in duplicate.

3.2.6. Enzymatic activities

Cellulase and xylanase quantification was determined using carboxymethylcellulose (CMC) (20 g L^{-1} in citrate buffer 0.05 N at a pH 4.8) and xylan (10 g L^{-1} in citrate buffer 0.05 N at a pH 4.8) as substrate, respectively. Release of reducing sugars after enzymatic hydrolyses was quantified using the 3,5-dinitrosalicylic acid (DNS) method as previously described.¹⁰

β -glucosidase quantification was assessed using p-nitrophenyl- β -D-glucopyranoside (PNG) as substrate.¹⁰ One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of p-nitrophenol per 1 minute at 50 °C and pH 4.8. Values of β -glucosidase activity were expressed in units per gram of dry solid (U g^{-1}).

Protease activity was measured using azo casein (5 g L^{-1} in sodium acetate buffer 50 mM, pH 5.0) as substrate.¹⁰ One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of azopeptides in 1 minute at 37 °C and pH 5.0. Values of protease activity were expressed in units per gram of dry substrate (U g^{-1}).

3.2.7. Nutritional parameters

Crude protein of solid residues was estimated using a defined factor of 6.25, after analysis of nitrogen content by the Kjeldahl method.²⁵

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were estimated after quantification of the organic constituents such as cellulose, hemicellulose, and Klason lignin by quantitative acid hydrolysis (QAH) in a two-stage acid treatment.²⁶

3.2.8. Statistical analysis

Differences in experiments among the same OC were analyzed using a one-way analysis of variance (ANOVA) at a significance level of 5%. All dependent variables were compared by a *post hoc* Tukey's Honestly Significant Difference (HSD) test or Least Significant Difference (LSD) *post hoc* test, using Statgraphics Plus 5.1 (Manusgistics, Inc., Rockville, MD).

3.3. Results and discussion

3.3.1. Enzymes produced by SSF

Compared to single cultures, the use of fungal consortia is an interesting approach for OC bioprocessing due to the production of different enzymes by different species with concomitant impacts on the obtention of other bioproducts and on the final composition of the bioprocessed OC. In this work, three filamentous fungi, *R. oryzae*, *A. ibericus*, and *A. niger*, were selected to explore the effect of using different fungal consortia of two species in SSF of two different OC, SFC and RSC.

The enzyme activities produced by each fungal consortia using SFC and RSC as substrate are presented in **Figure 3.1**. A significantly improved performance of enzyme production by the consortium *R. oryzae* + *A. ibericus* was observed for almost all enzymes monitored except for xylanases in SFC. As regards xylanase, slight differences were observed between co-cultures but the consortia *A. niger* + *R. oryzae* produced the highest enzymatic activity (**Fig. 3.1.B**). Maximum cellulase (**Fig. 3.1.A**) produced by the consortium *R. oryzae* + *A. ibericus* was 1.32 and 1.90-fold higher than the obtained by *A. ibericus* + *A. niger* and *A. niger* + *R. oryzae* consortia, respectively. It is worth stressing that cellulase activities obtained by all fungi co-cultures were higher than previously reported with single species in SFC using identical SSF conditions.¹⁰ *R. oryzae* + *A. ibericus* consortium stood out regarding β -glucosidase activity (**Fig. 3.1.C**), producing nearly 265 U/g that is 3.49-fold and 3.39-fold higher than the one obtained using *A. ibericus* + *A. niger* and *A. niger* + *R. oryzae* consortia, respectively. These values are also higher than those previously reported for monocultures.¹⁰ Similarly, protease production (**Fig. 3.1.D**) followed the same pattern with a maximum activity obtained by the consortium *R. oryzae* + *A. ibericus*, corresponding to a 2-fold higher enzymatic production compared with the other co-cultures. These results contrast with reported findings for monocultures, where *A. niger* was globally the best producer of enzymes by SSF using SFC,¹⁰ revealing that a microbial consortium of weaker enzyme producers in a specific substrate may allow obtaining similar results than the attained with monocultures.

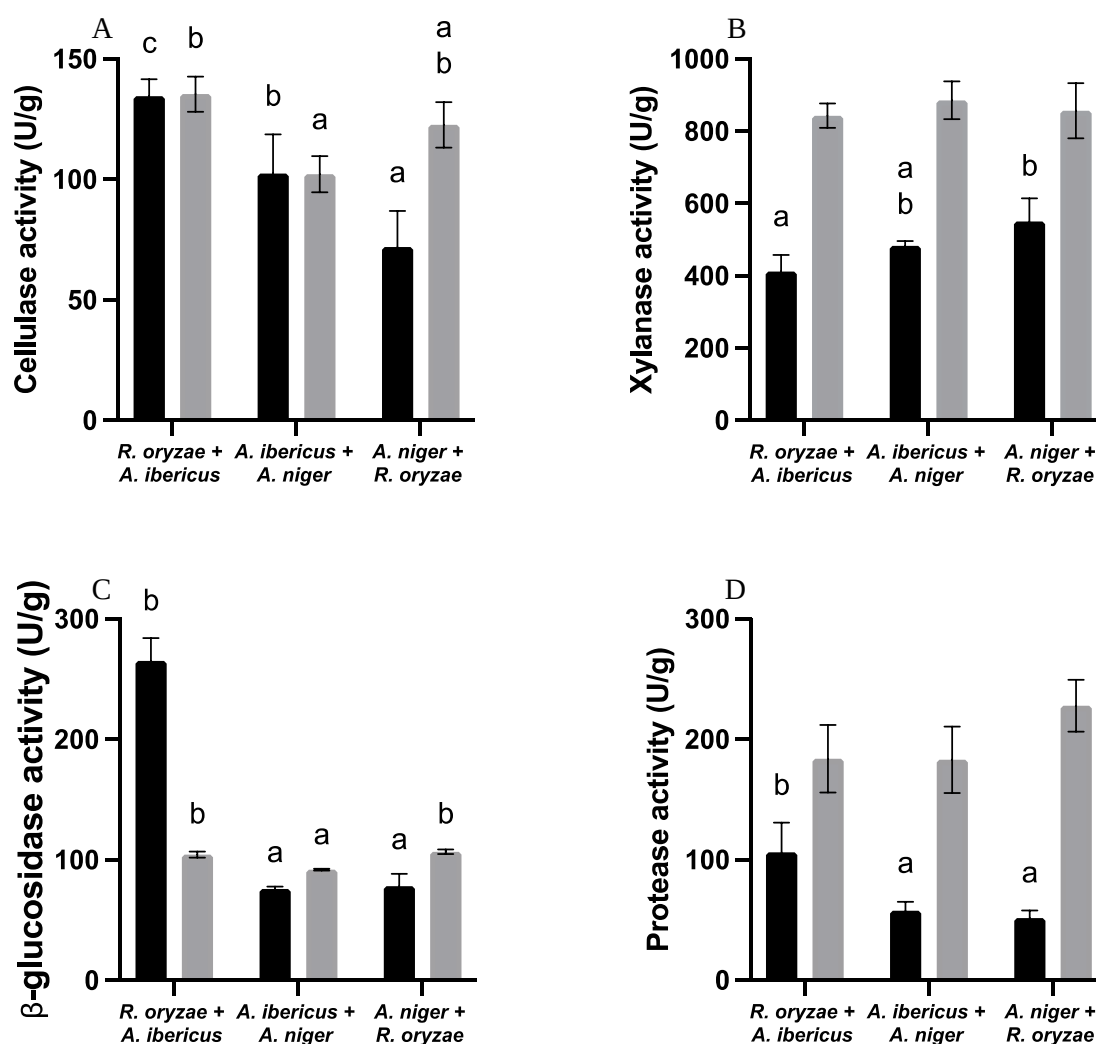


Figure 3.1. Activity of extracellular enzymes, cellulases (**A**), xylanases (**B**), β -glucosidases (**C**), and proteases (**D**) during SSF of SFC (black) and RSC (grey) using fungal consortia. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters for each substrate are not statistically different (Tukey test; $P < 0.05$).

The produced enzyme's activities by SSF using RSC as substrate were quite similar for all consortia. Maximum cellulase production (**Fig. 3.1.A**), was nearly 140 U/g in SSF of RSC by *R. oryzae* + *A. ibericus* consortium that is 1.32-fold higher ($P < 0.05$) than the produced by *A. ibericus* + *A. niger*. Also in RSC, it is worth noticing that cellulase activities obtained in SSF by all consortia were above the previously reported values obtained with pure cultures of each species in SFC and RSC.¹⁰ Also, xylanase activities (**Fig. 3.1.B**) obtained in the herein reported work were higher than the values previously reported, where *A. niger* was the best producer.¹⁰ The fungi consortium, *A. ibericus* + *A. niger*, produced

the maximum xylanase activity of 886 U/g but the other consortia were able to achieve comparable values. The activity of β -glucosidase (**Fig. 3.1.C**) follows the same pattern as cellulase with *R. oryzae* + *A. ibericus* alongside *A. niger* + *R. oryzae* consortia being the highest producers ($P < 0.05$). *R. oryzae* was previously reported as the best β -glucosidase producer in SSF of RSC.¹⁰ All co-cultures produced similar values of protease activity (**Fig. 3.1.D**), which were about 4-fold higher than the reported values for monocultures of these species.¹⁰

Overall, the consortium *R. oryzae* + *A. ibericus* showed the highest carbohydrases and proteases production efficiency, using SFC or RSC as substrate. These data highlight that in SSF, the behavior of fungal consortia, regarding enzyme production, cannot be predicted according to the behavior of single cultures.

The benefits of microbial consortia for the production of enzymes were reported by other authors that obtained higher enzymatic production by SSF using microbial co-cultures when compared to monocultures. Oliveira Rodrigues et al.¹⁸ tested five fungal strains in SSF experiments using raw sugarcane bagasse and wheat bran (1:1). The authors reported maximum production of β -glucosidase of 171 U/g using a consortium of *A. niger* SCBM01, *Trametes versicolor* 561, and *Pleurotus ostreatus* PL06, which corresponds to a 2-fold increase compared to the maximum obtained using monocultures. Cellulase production (CMCase), presented similar values of enzyme activity between microbial consortium (73.32 U/g) and monocultures (82.70 U/g). Maehara et al.²¹ also tested the effect of monocultures over co-cultures using steam-exploded sugarcane bagasse and wheat bran (1:1) as substrate for SSF. The co-cultivation of *Trichoderma reesei* with *A. niger* produced the highest amounts of β -glucosidase (64.5 U/g) and xylanase (539.7 U/g). The use of cottonseed as substrate for SSF was exploited by Grewal et al.²⁰, using mono and co-cultures. The highest production of cellulase (CMCase) and β -glucosidase was 155.41 U/g and 29.73 U/g, respectively. Maximum values were achieved using a consortium of *A. niger* (NRRL 3122), *T. reesei* (NRRL 6156), and *Phanerochaete chrysosporium* (MTCC 787). Santos et al.¹⁹ studied the consortium of *A. niger* SCBM1 and *A. fumigatus* SCBM6 using sugarcane bagasse and wheat bran as substrate for SSF. The authors reported maximum xylanase and β -glucosidase activity of 375 U/g and 80 U/g, respectively.

Protease production by SSF using fungi consortia has been less studied and available data with monocultures showed lower activity values than the ones herein reported. Oberoi et al.²⁷ reported a maximum protease activity of around 40 U/g using a consortium of *A. niger* and *T. reesei*, using kinnow waste and wheat bran in a ratio of 4:1. Castro and Sato,²⁸ reported maximum production of protease of

59.87 U/g by *A. oryzae* LBA01, using wheat bran as substrate for SSF; and Gupta et al.²⁹ reported maximum protease production of 52.5 U/g by *A. niger*, using mahuca cake as substrate in SSF.

Experimental data regarding the production of lignocellulolytic and proteolytic enzymes obtained with this work are clearly above the average reported values in literature **Table 3.1.** The use of microbial consortia is a viable and clean alternative for the exploitation of lignocellulosic materials to produce enzymatic cocktails, without the addition of external supplements. Microbial consortia exist within nature establishing symbiotic relations and, in this case, the results of the positive interactions are evident, resulting in an increased enzymatic production over monocultures.

3.3.2. Antioxidant potential of fermented extracts of oilseed cakes

Extracts of fermented SFC and RSC were analyzed to assess TPC and antioxidant properties (**Fig. 3.2.**). Maximum extraction of TPC from SFC was observed in fermented extracts obtained from the consortium *R. oryzae* + *A. ibericus* (**Fig. 3.2.A**), where TPC was 4.84 and 1.83-fold higher ($P < 0.05$) than the obtained with *A. ibericus* + *A. niger* and *A. niger* + *R. oryzae* consortia, respectively. Additionally, only *R. oryzae* + *A. ibericus* consortium was able to outpace the concentration of TPC obtained by the control. Previous work by Sousa et al. reported higher extraction of TPC from fermented OC by *R. oryzae* monoculture in comparison to *A. ibericus* and *A. niger*. This fact may explain the higher values of TPC as a result of SSF with *R. oryzae* + *A. ibericus* and *A. niger* + *R. oryzae* consortia compared to *A. ibericus* + *A. niger* consortium. Moreover, the action of lignocellulolytic enzymes is well described as a key factor influencing the extraction of phenolics from lignocellulosic fractions.^{30–32} The action of lignocellulolytic enzymes, namely β -glucosidase, on OC matrix allowed the release of water-soluble phenolic compounds. The highest production of this enzyme observed in *R. oryzae* + *A. ibericus* consortium led to a higher concentration of TPC. In fact, the enzymatic profile observed for β -glucosidase presents similarities to the TPC profile. Variations in these profiles may be attributed to the action of other enzymes. Extracts of fermented RSC using fungi consortia had similar concentrations of water-soluble phenolics. *A. niger* + *R. oryzae* consortium stood out, showing the maximum concentration of TPC with statistically significant differences ($P < 0.05$) over control.

Table 3.1. Maximum enzymatic activities obtained by SSF using fungal consortia.

Consortia	Solid substrates	Enzyme	Activity (U g ⁻¹)	Reference
<i>Aspergillus niger</i> <i>SCBM1</i> + <i>Trametes versicolor</i> 561 + <i>Pleurotus ostreatus</i> PL06	Raw sugarcane bagasse + raw wheat bran (1:1)	Carboxymethylcellulase (CMCase) β -glucosidase	73.3 171.1	18
<i>Trichoderma reesei</i> + <i>A. niger</i>	Steam-exploded sugarcane bagasse + wheat bran (1:1)	β -glucosidase Xylanase	64.5 539.7	21
<i>Aspergillus niger</i> + <i>Trichoderma reesei</i> + <i>Phanerochaete chrysosporium</i>	Cottonseed cake	CMCase β -glucosidase	155.4 29.7	20
<i>Aspergillus niger</i> <i>SCBM1</i> + <i>Aspergillus fumigatus</i> SCBM6	Sugarcane bagasse + wheat bran (1:1)	Xylanase β -glucosidase	375 80	19
<i>Aspergillus niger</i> + <i>T. reesei</i>	Kinnow waste + Wheat bran (4:1)	Protease	40	27
Maximum enzymatic activity values reported in this work				
<i>R. oryzae</i> + <i>A. ibericus</i>	SFC and RSC	Cellulase	135	This work
<i>A. ibericus</i> + <i>A. niger</i>	RSC	Xylanase	886	
<i>R. oryzae</i> + <i>A. ibericus</i>	SFC	β -glucosidase	265	
<i>A. niger</i> + <i>R. oryzae</i>	RSC	Protease	228	

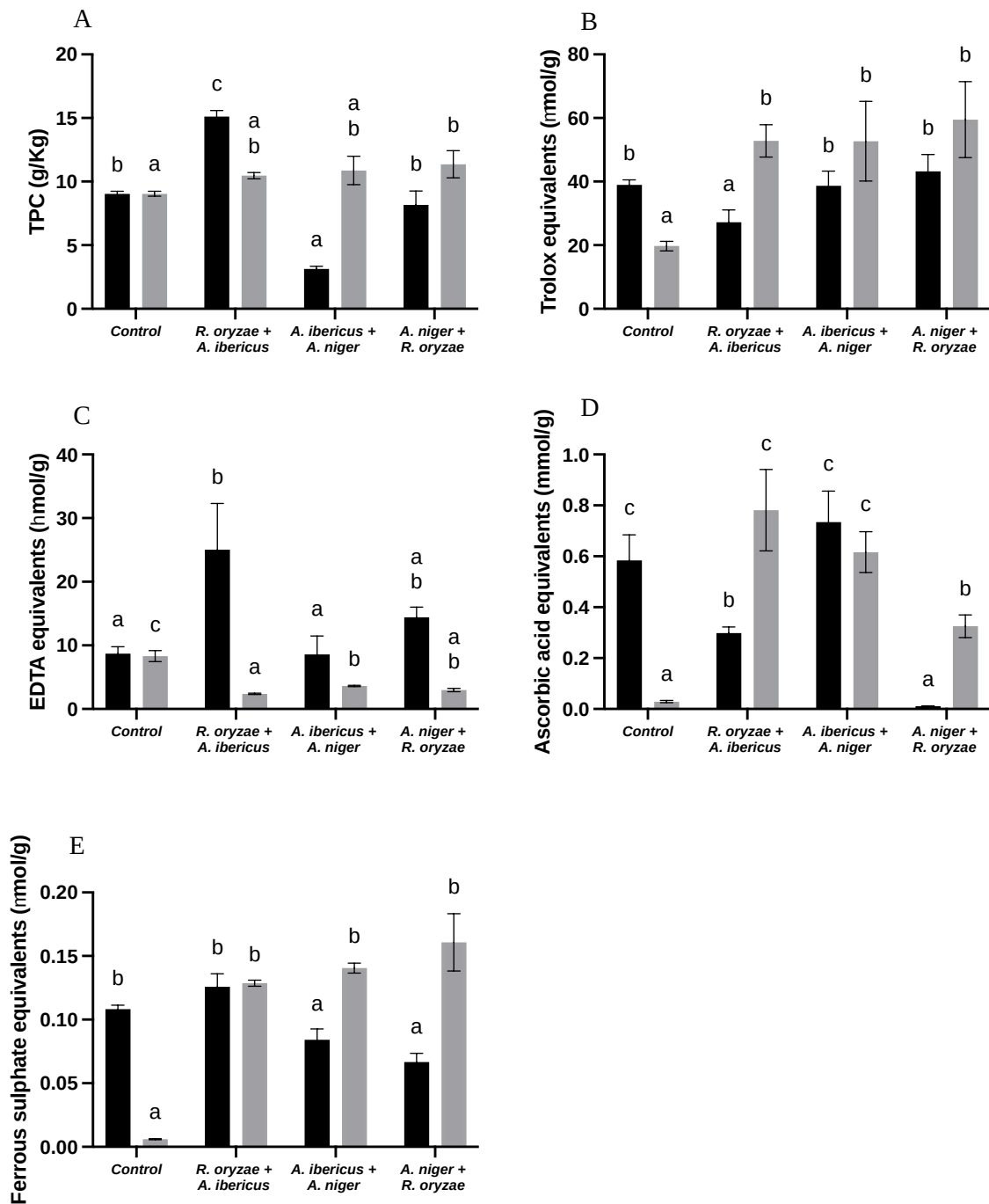


Figure 3.2. Total phenolic content (TPC) and antioxidant potential of aqueous extracts of controls (oilseed cake sterilized) and fermented SFC (black) and RSC (grey) using fungal consortia. **(A)** TPC; **(B)** DPPH radical scavenging activity; **(C)** iron chelation ability; **(D)** superoxide radical scavenging activity; **(E)** reducing ability. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters for each substrate are not statistically different (Tukey test; $P < 0.05$)

Figure 3.2.B-E depicts the results of four different antioxidant assays used to assess the antioxidant potential of fermented SFC and RSC. Fermented extracts of SFC obtained by consortia *A. ibericus* + *A. niger* and *A. niger* + *R. oryzae* exhibited a highest scavenging potential of free radicals (**Fig. 3.2.B**) over *R. oryzae* + *A. ibericus* consortium. However, these consortia were not able to outpace the scavenging potential of free radicals observed in the control.

On the other hand, the scavenging potential of free radicals was successfully increased throughout SSF using RSC as substrate. An increased scavenging potential of 2.79-fold over control ($P < 0.05$) was observed for the three consortia.

Iron chelators may act as antioxidants by their action in scavenging reactive oxygen species (ROS) but also by reducing the quantity of available iron that undergo Fenton reactions, resulting in a decreased amount of generated hydroxyl radical ($\bullet\text{OH}$).³³ The highest ability to chelate iron of extracts of fermented SFC (**Fig. 3.2.C**) was obtained by *R. oryzae* + *A. ibericus* consortium. This extract presented a chelation potential 2.78-fold higher than *A. ibericus* + *A. niger* consortium and 1.79-fold compared to *A. niger* + *R. oryzae* culture. Despite an increase of the chelation potential of nearly 36 % of *A. niger* + *R. oryzae* extracts over control, no statistically significant differences were observed. *A. ibericus* + *A. niger* consortium fermented extracts of RSC achieved maximum chelation ability, using this substrate. However, the chelation potential obtained by the consortia was between 2 and 3.2-fold lower than control. This fact may be explained by fungal interactions and consequent inhibitions. Previously, Sousa et al.¹⁰ reported the increase of chelation power of fermented extracts of RSC using monocultures. Additionally, extracts antioxidant potential may be related to antioxidants produced by the fungi itself. Consortia where *A. niger* was present led to extracts with similar or higher chelation potential. The work of Sousa et al.¹⁰ reported a maximum chelation potential obtained by *A. niger* when compared to *R. oryzae* and *A. ibericus* monocultures.

Superoxide anion is one of the main ROS and it is very difficult to maintain this anion stable for a long period. It is related to several harmful biological processes such as protein denaturation and lipid peroxidation.³⁴ The scavenging of superoxide anion (**Fig. 3.2.D**), exhibited by fermented extracts of SFC from *A. ibericus* + *A. niger* consortium achieved similar values as the control. Still, this consortium obtained a scavenging potential of 2.33 and 70-fold higher than *R. oryzae* + *A. ibericus* and *A. niger* + *R. oryzae* consortia, respectively. Extracts from *A. niger* + *R. oryzae* consortium had scavenging potential almost null. *A. ibericus* may be the key factor regarding this antioxidant activity maintaining a symbiotic relationship with the other fungi and explaining the low values obtained by *A. niger* + *R. oryzae* consortium. In previous work, Sousa et al.¹⁰ reported a higher scavenging potential of SFC

extracts fermented by monocultures of *A. niger* and *A. ibericus* when compared to *R. oryzae* monocultures using the same substrate. The presence of the fungi *A. ibericus* in co-cultures using RSC as substrate led to a higher scavenging potential over *A. niger* + *R. oryzae* consortium. No statistically significant differences ($P < 0.05$) were observed between *R. oryzae* + *A. ibericus* and *A. ibericus* + *A. niger* consortia. These consortia were, respectively, responsible for an increase of nearly 27 and 21-fold over control. Although with a lower increase, *A. niger* + *R. oryzae* consortium was able to significantly increase the scavenging potential of superoxide anion by about 11-fold over control.

The reduction of oxidants is a path to minimizing their harmful effects related to oxidative stress. Additionally, the iron reduction from Fe^{3+} to a more stable form, Fe^{2+} , interrupts the oxidation chain.

Figure 3.2.E depicts the reduction power of SFC and RSC fermented extracts. SSF using the consortium *R. oryzae* + *A. ibericus* resulted in a 17% increase of reduction potential over control and corresponds to 1.6 and 1.86-fold higher values than the obtained with *A. ibericus* + *A. niger* and *A. niger* + *R. oryzae* consortia. On the other hand, all extracts of fermented RSC by all consortia had similar reduction potential. SSF had a clear positive effect on this antioxidant activity using RSC as substrate, resulting in around 21, 24 and 27-fold enhancement over the control, for *R. oryzae* + *A. ibericus*, *A. ibericus* + *A. niger*, and *A. niger* + *R. oryzae* consortia, respectively.

Generally, it was proven the potential of SFC and RSC as substrate of SSF to produce bioactive compounds with antioxidant potential, using fungal consortia. Few studies have been found regarding the use of fungal consortia to produce extracts with proven *in vitro* antioxidant potential. Abd Razak et al.³⁵ studied the interaction of *R. oligosporus* and *Monascus purpureus* to enhance the phenolic content and antioxidant potential of rice bran. The authors reported nearly a 5-fold increase of TPC and the reduction potential of fermented extracts. On the other hand, the authors did not observe statistically significant differences among results of scavenging potential of free radicals. Within the analysis of antioxidant potential assays, differences between each assay represent the specificity of each method. DPPH method measures the scavenging potential of free radicals while superoxide assay measures the extracts' capacity to scavenge the superoxide anion. ICA method is based on the chelation potential of extracts while FRAP measures their reducing ability. Different patterns between antioxidant assays were observed in this work and were depicted in **Figure 3.2**. Also, the TPC of extracts is not correlated with their antioxidant potential. It is known that fungal hydrolytic enzymes such as β -glucosidase and xylanase, may increase TPC and antioxidant potential.³⁶ These enzymes act directly on the substrate leading to an increase in the availability of free hydroxyl groups on phenolic structures. This action results in increased content of free phenolic groups and consequently an increase of the antioxidant

potential of the substrate. Additionally, hydrolysis of phenolic conjugates may release free phenolic compounds and low molecular weight molecules with higher antioxidant potential.³²

Chen et al.³⁷ tested a microbial consortium of the fungus *Monascus anka* and the gram-positive bacteria *Bacillus subtilis* for the enhancement of phenolic fractions liberation from oats. The author reported a 23.7-fold increase of TPC after 12 days of fermentation and a reduction of more than 50% of the IC₅₀ value resulting in an increment of antioxidant potential when compared to unfermented oats.

SSF with specifically fungal consortia is worth exploration due to the symbiotic relations established by microorganisms. However, in some cases may happen some type of inhibition between species. Sousa et al.¹⁰ reported a higher antioxidant potential of monocultures fermented extracts using the same species as the ones present in these consortia. *A. niger* monocultures led to extracts with a better overall response regarding the different antioxidant assays while *R. oryzae* to the highest reported values of TPC. Also, a higher chelation potential of fermented RSC with *A. niger* monoculture was reported compared to the herein obtained values, but extracts obtained with fungal consortia showed a reducing potential 2.2 and 1.3-fold higher than the maximum values reported for monocultures.

3.3.3. Nutritional parameters of fermented oilseed cakes

Crude protein and fiber fractions including cellulose, hemicellulose, and lignin, are important nutritional parameters in animal feed. **Table 3.2.** shows crude protein and fiber fractions of fermented SFC and RSC.

SFC and RSC are protein-rich substrates exhibiting a content of around 400 g/kg of protein per dry mass of solid. During SSF of SFC, fungal consortia did not significantly affect the amount of crude protein. On the other hand, RSC crude protein was significantly increased during SSF by the *Aspergillus* species consortia. The work of Sousa et al.¹⁰ reported a decrease of crude protein after SSF of SFC and RSC, using monocultures of the same species as in this work. The ability of fungi consortia to maintain or increase the crude protein of OC's shows that the interaction between species overcomes monoculture limitations. According to INRAE et al.³⁸ feed tables, fermented SFC and RSC present crude protein values within the range recommended for animal feed, even exceeding some common SFC and RSC values. The fiber fractions of OC's were significantly affected during SSF. Unlike ruminants, in monogastric nutrition, dietary fiber is represented by carbohydrates present in plant cell walls that are indigestible by endogenous enzymes.³⁹ Particularly, cellulose and lignin fractions decrease feed digestibility as they are the least usable fractions of crude fiber and due to the production of lignocellulosic enzymes in SSF, a decrease in these fractions was expected.

Table 3.2. Nutritional composition of sunflower cake and rapeseed cake. The mean difference of values with different letters is significantly different, following a One-way ANOVA with LSD Post Hoc Test, $P < 0.05$.

		Sunflower cake		
Composition (g Kg ⁻¹)	Control	<i>R. oryzae</i> + <i>A. ibericus</i>	<i>A. ibericus</i> + <i>A. niger</i>	<i>A. niger</i> + <i>R. oryzae</i>
Crude Protein	413 ± 4	387 ± 36	371 ± 14	414 ± 8
Cellulose	112 ± 8 ^a	114 ± 1 ^a	119 ± 9 ^{ab}	137 ± 10 ^b
Hemicellulose	90 ± 7 ^b	69 ± 8 ^a	71 ± 9 ^{ab}	75 ± 6 ^{ab}
Lignin	116 ± 5 ^a	172 ± 0 ^c	175 ± 17 ^c	147 ± 23 ^b
		Rapeseed cake		
Composition (g Kg ⁻¹)	Control	<i>R. oryzae</i> + <i>A. ibericus</i>	<i>A. ibericus</i> + <i>A. niger</i>	<i>A. niger</i> + <i>R. oryzae</i>
Crude Protein	420 ± 7 ^a	441 ± 14 ^{ab}	453 ± 10 ^b	444 ± 6 ^{ab}
Cellulose	104 ± 3 ^a	125 ± 3 ^b	118 ± 6 ^b	96 ± 5 ^a
Hemicellulose	93 ± 1 ^c	54 ± 3 ^b	47 ± 3 ^{ab}	41 ± 4 ^a
Lignin	149 ± 5 ^a	199 ± 0 ^b	213 ± 17 ^b	227 ± 23 ^b

Hemicellulose is a polysaccharide of the plant cell wall. It surrounds cellulose microfibrils and therefore, it comprises the exterior part of the lignocellulosic materials, which is more accessible to microorganisms. Compared to controls, there was a reduction in hemicellulose fractions of 23% in SSF (statistically significant by LSD Post Hoc test with $P < 0.05$) by *R. oryzae* and *A. ibericus* consortium, using SFC as substrate. The consortia *A. ibericus* and *A. niger* and *A. niger* and *R. oryzae* were able to respectively reduce hemicellulose by 21% and 17%, however without statistically significant differences. The effect of microbial fermentation in the decrease of hemicellulose was even higher when RSC was used as substrate. Maximum reduction was obtained by *A. niger* and *R. oryzae* consortium achieving 56% of reduction, followed by *A. ibericus* and *A. niger* consortium with 49% reduction and, *R. oryzae* and *A. ibericus* with 42% (all reductions are statistically significant by LSD Post Hoc test with $P < 0.05$). As regards cellulose content, microbial degradation through SSF unaltered or increased cellulose concentration. Consequently, the cellulose content of fermented OC was equal or slightly higher than the values on the control on a dry matter basis. These results are in consonance with enzymes activity where was observed higher enzymatic activity of xylanase in fermented extracts, compared to cellulase. Xylanase can break down hemicellulose and this enzyme is produced in larger quantities.

Lignin, the most recalcitrant component of lignocellulosic biomass, was not degraded throughout SSF. Therefore, the final concentration of this component significantly increased in the final fermented biomass on a dry matter basis in both SFC and RSC, due to the degradation of the other fibrous fractions by fungal enzymes during SSF. The presence of high lignified cell wall components can result in a decrease in the nutritive value of the fermented feed. However, a proportion of insoluble fiber is necessary for animal feeding to enhance gut health and intestinal passage rate. Overall, lignin concentrations in fermented OC were within the values observed in other feed ingredients such as cereal by-products according to INRAE et al.³⁸

All species used in this work are generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA), thus their use for the degradation of lignocellulosic materials does not entail any obstacle for the use of the fermented OC as animal feed.

3.4. Conclusions

Synergistic interactions between fungal species in SSF were demonstrated, resulting in an increased performance to produce enzyme rich extracts with an additional property that is their high antioxidant potential, with great interest for many applications such as feed, food, cosmetics, and pharmaceutical industries. Additionally, despite the action of lignocellulosic enzymes produced by fungal consortia was mainly reflected on hemicellulose reduction, but fermented OC fibrous fractions are within the parameters used in the feed industry.

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Chapter 4

Biotechnological valorization of oilseed cakes: substrate optimization by simplex centroid mixture design and scale-up to tray bioreactor

This chapter is based on the following original research article:

Sousa, D.; Salgado, J. M.; Cambra-López, M.; Dias, A.; Belo, I. Biotechnological Valorization of Oilseed Cakes: Substrate Optimization by Simplex Centroid Mixture Design and Scale-up to Tray Bioreactor. *Biofuels, Bioprod. Biorefining* **2022**. <https://doi.org/10.1002/bbb.2428>.

Abstract

Sunflower (SFC), rapeseed (RSC), and soybean (SBC) cakes are examples of oilseed cakes (OC) used in animal feed. Bioprocessing of these OC by solid-state fermentation (SSF) aims to boost OC applications in feed and other industries by reducing antinutritional factors and releasing enzymes and antioxidants. A simplex centroid design was performed to optimize the substrate composition of SFC, RSC and SBC mixtures that maximize lignocellulolytic enzymes production by SSF with *Aspergillus niger* and, consequently improve the nutritional properties of OC. Enzymes production by SSF of OC mixtures exceeded the activity values obtained using single OC. A mixture composed by 50 % (w/w) RSC and SBC was found as the optimum substrate. A scale-up from flasks to tray-type bioreactors demonstrated SSF reproducibility leading to the production of 299 U g⁻¹ cellulase, 1476 U g⁻¹ xylanase, 191 U g⁻¹ b-glucosidase, and 220 U g⁻¹ protease. The obtained enzymatic extract also presented antioxidant activity (around 50 mmol Trolox equivalents/g). This study demonstrated that bioprocessing of OC mixtures through SSF is an effective approach to obtain value-added products with applications as feed or feed additive and to increase the liberation of bioactive compounds with applications in the feed, food, cosmetic or pharmaceutical industries.

Keywords: simplex centroid design, oilseed cakes, OC mixtures optimization, scale-up, enzymes, antioxidants

4.1. Introduction

Production chains improvement, valorization of by-products, and their reintroduction into the market according to the concept of circular economy are key steps that improve economy and contribute to a sustainable environmental development. In a near future protein supply will be a limiting factor, so it is essential to find alternatives to common sources of supply for either animal or human nutrition.

Vegetable oils have one of the highest trade shares (41 %) of production among all agricultural commodities.¹ They are mainly applied in the food sector, oleochemical, and biodiesel industries.

Soybean is the largest produced oilseed on a global scale being the United States, Brazil, and Argentina the major world producers. In 2021, roughly 373 million tons of this commodity were produced, and projections show that its products will continue to rise, with the possibility to achieve approximately 411 million tons by 2030.¹ However, concerns with soy production sustainability have arose over the last years due to intensive production which is related with non-rotation crops, deforestation, and loss of native vegetation. According to Fraanje and Garnett,² nearly 75 % of produced soybean is used to feed animals, being the poultry sector the major consumer of processed soybean (53 %) amongst all livestock and poultry species. According to the Organization for the Economic Co-operation and Development and the Food and Agriculture Organization (OECD/FAO) Agriculture Outlook for 2021-2030, production of rapeseed, sunflower seed, and groundnuts will continue to rise. These crops are among the largest produced, after soybean. In 2021, around 159 million tons of these seeds were produced, and by 2030 the forecast is to achieve roughly 179 million tons.¹

Generally, oilseeds are processed (90 % of soybeans and 87 % of other oilseeds, including rapeseed and sunflower) to obtain the vegetable oil. The extraction process leads to the obtainment of large quantities of solid by-products called cakes. Oilseed cakes (OC) are largely produced every year. In 2021, world production of this commodity achieved 363 million tons with an average trading price of 391.01 US dollars per ton.¹ In practice, OC are commonly used as animal feedstuff, plant fertilizer, or soil compost.^{3,4} Additionally, they can be used for energy production or protein concentrates. Nonetheless, the use of OC as animal feed is the major application, it presents some limitations due to the presence of antinutritional factors and limitations regarding its concentration of sulphur containing amino acids in the case of soybean seeds and rapeseed, and lysine in the case of sunflower seeds.⁵

OC are characterized by a high protein content, the presence of non-starch polysaccharides, fibers, antioxidants, lipids, minerals, and vitamins.^{6,7} Independently of the use of organic solvents after mechanical extraction of the vegetable oils, some polar compounds such as phenolic acids, lignans, or flavonoids with antioxidant potential remain in the OC.⁸ These bioactive compounds can be extracted,

concentrated, and incorporated in foods and feed as additives providing additional protection against oxidative stress. The protein content of sunflower cake (SFC), rapeseed cake (RSC), and soybean cake (SBC) account for 14-46 % (w/w) of dry weight while fiber varies between 5 and 37 %.^{9,10} Additionally, OC have residual concentrations of phenolic compounds ranging from 3 to 12 g kg⁻¹ with antioxidant properties allowing them to act as radical scavengers, chelators, or reduction agents.⁹ The employment of these OC in low-cost and environmental impact technologies such as bioprocess allows the valorization of this biomass contributing to the improvement of vegetable oils production chains.

Solid-state fermentation (SSF) is a fermentation process in which microorganisms grow in the absence or near absence of free water where a solid substrate is used as the carbon source for microorganisms' growth. This fermentation mimics the conditions observed in natural habitats and filamentous fungi are the microorganisms that better adapt to it.¹¹ Several by-products and agro-industrial wastes have been employed in SSF for the production of valuable compounds such as cellulase, xylanase, and phenolic compounds with antioxidant potential while simultaneously increasing the nutritional properties of the final products.^{9,12-15} The new value-added products can be further incorporated into the trade market contributing to the implementation of the circular economy concept. One of the main drawbacks when considering bioprocessing is the unbalanced nutrients of the substrate which may limit the growth of microorganisms. Thus, the mixture of substrates can overcome this limitation promoting fungal growth while simultaneously increasing the production of bioactive compounds.¹⁶

Filamentous fungi are natural degraders of lignocellulosic biomass as they produce extracellular lignocellulolytic enzymes.¹⁷ Additionally, they can develop hyphae that penetrate the solid matrix of substrates. The depolymerization of lignocellulosic biomass by these microorganisms is mainly focused on hemicellulose and cellulose that release fermentable sugars able to promote fungal growth.¹⁸ On the other hand, some species of filamentous fungi are more prone to deconstruct the recalcitrant matrix of lignin, allowing the release of bioactive compounds with antioxidant potential.¹⁷ Antioxidants are important compounds involved in the prevention of oxidative stress, caused by reactive oxygen species (ROS). ROS can induce harmful effects including damaging and altering the DNA structure, nucleic acids, lipids, and proteins.¹⁹ The produced valuable bioactive compounds namely enzymes found applications in industries such as textile, paper, food, and feed while compounds with potential bioactivities (antioxidant) can be incorporated into foods, used as animal feed additives, or applied in the cosmetics and pharmaceutical industries.

This work aimed to study the bioprocessing of mixtures of OC for obtaining value-added products in the concept of biorefinery contributing to the implementation of the circular economy. In this sense,

different mixtures of OC were evaluated as substrate for SSF to produce cellulase, xylanase, β -glucosidase, and protease and concomitantly liberate phenolic compounds with antioxidant potential. Additionally, the effect of SSF on substrate composition (crude protein and fiber) was evaluated. To assess the optimum mixture of substrates, a simplex centroid mixture design was used. The use of statistical mixture designs has the advantage of a clear representation of effects and interactions between different components in a certain response.²⁰ SSF at optimal conditions was scaled-up to tray-type bioreactor.

4.2. Materials and methods

4.2.1. Agro-food by-products

Three OC from the vegetable oils industry were used during this work: sunflower cake (SFC), rapeseed cake (RSC), and soybean cake (SBC). OC were obtained after cold-press extraction of oil and supplied by industries operating in Portugal. SFC was provided by Sorgal, S. A. while RSC and SBC were provided by IBEROL – *Sociedade Ibérica de Oleaginosas*, SARL. OC were dried at 65 °C for 24 hours and stored at 20 °C in hermetic bags. Previously characterized OC by Sousa et al. have high protein contents ranging from 40 to 50 % (w/w), variations on lignocellulosic composition, particularly in lignin ranging from 2 to 8%, and the presence of small fractions of phenolic compounds.⁹

4.2.2. Microorganisms

Aspergillus niger CECT 2915, obtained from CECT (“Colección Española de Cultivos Tipo”, Valencia, Spain) was used in this work. The fungus was selected according to its potential to improve the nutritional value of OC and simultaneous production of commercially relevant enzymatic extracts with antioxidant potential, reported by Sousa et al.⁹ Fungi were preserved in glycerol at -80 °C and revived on potato dextrose agar (PDA) plates (39 g L⁻¹). Cultures were stored at 4° C for a maximum of three months and subcultured in PDA slants. PDA slants were incubated at 25 °C for 7 days and used as inoculum for SSF.

4.2.3. Solid-state fermentation

The mixture of substrates was performed according to **Table 1**, following a simplex centroid mixture design. SSF of each experiment was carried out in 500 mL Erlenmeyer flasks with cotton caps to allow oxygen transfer. A total amount of 10 g of dry solid was used, moisture was adjusted to 75 % with

distilled water (w/w, wet basis). Flasks were sterilized for 15 min at 121 °C. For inoculation of *A. niger* into the different substrate mixtures, a sterile solution of peptone composed of 1 g L⁻¹ peptone and 0.1 g L⁻¹ Tween-80 was used to recover spores from PDA slants and to prepare an inoculum suspension with a concentration of 10⁶ spores mL⁻¹, from which 2 mL was added to each flask. The SSF was carried out over 7 days at 25 °C in a controlled temperature chamber. Each experiment was performed in duplicate and a control experiment without inoculation was performed following the SSF conditions described.

4.2.4. Bioactive compounds extraction

At the end of SSF, distilled water was added to fermented OC in a solid/liquid ratio of 1:5 (w/w). Mixtures were stirred at 0.5 *g* for 30 min at 20 °C and then, filtered through a fine-mesh net. The liquid fraction was centrifuged at 2264 *g* for 10 min at 4 °C, to recover any remaining solid fraction. Supernatant (extracts) were recovered and stored at -20 °C until analysis.

4.2.5. Enzymatic activities

Quantification of enzymatic activities was performed following the methods described by Sousa et al. and all activities were expressed as units per gram of dry substrate (U g⁻¹).⁹

Cellulase and xylanase activity was evaluated using carboxymethylcellulose (CMC) (20 g L⁻¹ in citrate buffer 0.05 N at a pH 4.8) and xylan (10 g L⁻¹ in citrate buffer 0.05 N at a pH 4.8) as substrate, respectively. After enzymatic hydrolysis of substrates, the release of reducing sugars was quantified using the 3,5-dinitrosalicylic acid (DNS) method. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose or xylose, respectively for cellulase or xylanase, per minute at 50 °C and pH 4.8.

β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (pNG) as substrate. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute at 50 °C and pH 4.8.

Azo casein (5 g L⁻¹ in sodium acetate buffer 50 mM, pH 5.0) was used as the substrate for protease quantification. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of azopeptides in 1 minute at 37 °C and pH 5.0.

4.2.6. Chemical composition and nutritional parameters

The ash content was determined by high temperature treatment at 575 °C for 2 h in a muffle.

Nitrogen content of solid residues was quantified using the Kjeldahl method and estimation of crude protein was obtained using a defined factor of 6.25.²¹

The concentration of organic constituents namely cellulose, hemicellulose, and Klason lignin was obtained through quantitative acid hydrolysis in a two-stage acid treatment.²²

4.2.7. Total phenolic compounds and antioxidant activity

Total phenolic compounds (TPC) were quantified in extracts from fermented and unfermented OC using the Folin-Ciocalteu method (Commission Regulation (ECC) No. 2676/90), with some modifications.⁹ TPC was expressed as mg of gallic acid equivalents per g of dry matter.

The antioxidant potential of fermented extracts was evaluated using four *in vitro* antioxidant assays as described by Sousa et al.⁹ The scavenging potential of free radicals was evaluated by the DPPH method and expressed as micromoles of Trolox equivalents per gram of dry matter ($\mu\text{mol g}^{-1}$).²³ Extract's capacity to chelate ferrous ion was assessed by the iron chelating ability (ICA) assay and expressed as EDTA equivalents (nmol/g). Scavenging of superoxide radical was determined using the PMS-NADH non-enzymatic assay and expressed as micromoles of ascorbic acid equivalents per gram of dry matter ($\mu\text{mol g}^{-1}$).²⁴ Extracts reduction potential was evaluated by the Ferric Reducing Antioxidant Power Assay (FRAP) and expressed as ferrous sulfate equivalents ($\mu\text{mol/g}$).²⁵ All assays were performed in duplicate.

4.2.8. Experimental design and statistical analysis

The optimal mixture of OC's to be used as substrate for SSF was evaluated using a simplex centroid mixture design. This design allows the identification of synergistic or antagonistic effects through the mixture of solid substrates. Seven experiments were performed with all three independent variables (SFC, RSC, and SBC) at different concentrations (w/w): 100 %, 50 %, 33 %, and 0 %, to a final total amount of 10 g of dry matter, as shown in **Table 1**. All experiments were performed in duplicate and in each run, a control group was performed, without fungus inoculation. The dependent variables studied were cellulase, xylanase, b-glucosidase, protease, crude protein, cellulose, hemicellulose, and lignin.

Multiple regression analysis was applied to experimental data, obtaining the following equation (4.1.) that represents this model:

$$Y = b1.x1 + b2.x2 + b3.x3 + b12.x1.x2 + b13.x1.x3 + b23.x2.x3 + b123.x1.x2.x3 \quad (4.1.)$$

Where Y represents the response variable, b are the regression coefficients and x are the independent variables. Experimental data were evaluated using the Statistica software. To obtain a mixture of substrates that maximizes enzymes production and protein content, and also minimizes lignocellulosic components, an optimization by multiple response variables was carried out using Statgraphics Centurion software.

4.2.9. Scale-up process to tray-type bioreactors

SSF scale-up was validated in tray-type bioreactor, and the effect of headspace on dependent variables' responses was evaluated. Initially, the total amount of the optimum OC mixtures was increased from 10 g to 50 g of dry matter and two systems were compared: a 1 L Erlenmeyer flask (r= 5.05 cm; h= 17.6 cm) and a small tray (l= 15.20 cm; w= 10 cm, h= 5.3 cm). SSF was also performed in a tray bioreactor (l= 39.30 cm; w= 29.70 cm) with a final amount of dry matter of 400 g.

4.3. Results and discussion

4.3.1. Chemical composition of oilseed cakes and their mixtures

SSF efficiency can be affected by several parameters including pH, temperature, and aeration. The chemical composition of solid substrates is a critical factor when considering the production of bioactive compounds such as enzymes. The concentration of cellulose and hemicellulose may induce the production of lignocellulolytic enzymes while the protein concentration of a substrate may lead to the production of proteolytic enzymes such as protease.²⁶ Depending on the concerted action of the extracellular enzymes produced throughout SSF, these can degrade polysaccharides from the lignocellulosic matrix of OC into monosaccharides, oligosaccharides and low molecular weight polysaccharides releasing minerals and valuable phenolics with antioxidant potential properties. On the other hand, an initial high concentration of phenolics in the substrate may inhibit fungal growth and proliferation. A single OC as substrate may not be able to supply all the necessary nutrients to promote fungal growth while the mixture of substrates may overcome this limitation. Considering all these variables, substrate composition optimization is a critical step to increase bioprocess efficiency. Using a simplex centroid mixture design to optimize substrate composition for SSF, different substrate combinations of OC (**Table 4.1**) were selected, to evaluate their impact on microbial extracellular

enzymes (cellulase, xylanase, β -glucosidase, and protease) production and to obtain antioxidant potentially rich extracts. SBC has the highest crude protein content, being 1.3-fold higher than in the other two OC. Also, SBC has the highest cellulose and hemicellulose content. On the other hand, lignin content was highest in RSC and lowest in SBC. Lignin concentration of RSC was 3 and 6-fold higher than of SFC and SBC, respectively. The mixtures of SBC with the RSC and SFC, presented balanced composition of fibers and protein that may result in improved performance of the SSF of OC by the fungus. **Table 4.2** shows the results of the dependent variables obtained in each SSF with *A. niger*, according with the designed runs of the experimental matrix described in **Table 4.1**.

Table 4.1. Characterization of the solid substrate of each experiment of the simplex centroid mixture design.

Runs	Substrate (g)			Final composition in dry solid (g Kg ⁻¹)				
	SFC	RSC	SBC	Ash	CP	Cellulose	Hemicellulose	Lignin
1	10	0	0	84 $\pm$ 0	402 $\pm$ 6	143 $\pm$ 2	111 $\pm$ 7	83 $\pm$ 1
2	0	10	0	67 $\pm$ 2	398 $\pm$ 21	155 $\pm$ 16	136 $\pm$ 14	77 $\pm$ 17
3	0	0	10	63 $\pm$ 1	504 $\pm$ 14	162 $\pm$ 3	155 $\pm$ 7	22 $\pm$ 1
4	5	5	0	75 $\pm$ 1	400 $\pm$ 7	149 $\pm$ 9	124 $\pm$ 4	80 $\pm$ 9
5	5	0	5	73 $\pm$ 1	453 $\pm$ 10	152 $\pm$ 0	133 $\pm$ 0	52 $\pm$ 0
6	0	5	5	65 $\pm$ 2	451 $\pm$ 3	158 $\pm$ 7	146 $\pm$ 10	50 $\pm$ 8
7	3.33	3.33	3.33	70 $\pm$ 1	430 $\pm$ 0	151 $\pm$ 5	133 $\pm$ 5	60 $\pm$ 5

SFC, sunflower cake; RSC, rapeseed cake; SBC, soybean cake; CP, crude protein; TPC, total phenolic compounds; RS, reducing sugars.

The mixture of OC had a significant impact on the production of all enzymes resulting in increased production, compared to the use of single OC as substrate. Cellulase activity obtained in mixtures of two different OCs was around 3-fold higher than the obtained by SSF of single SBC and 6-fold higher than the obtained with single SFC and RSC. Mixtures of the three OCs led to the production of 2-fold more cellulase than with single SBC that was the single best substrate for cellulase production, which is in accordance with the highest content of cellulose of this OC. As regards other enzymes (xylanase, β -glucosidase, and protease), **Table 4.2** shows that the mixture of all OCs (run 7) led to the best activity values, showing the synergistic effects of OCs in the solid medium composition that allowed best performance of the fungus.

Table 4.2. Composition of solid substrates after solid-state fermentation and results of studied dependent variables using simplex centroid design.

Run	Xyl. (U g ⁻¹)	Cel. (U g ⁻¹)	β-gluc. (U g ⁻¹)	Prot. (U g ⁻¹)	CP (g Kg ⁻¹)	Cellulose (g Kg ⁻¹)	Hemi- cellulose (g Kg ⁻¹)	Lignin (g Kg ⁻¹)
1	398 ± 7	54 ± 0	209 ± 38	157 ± 19	396 ± 4	86 ± 25	34 ± 19	163 ± 3
2	692 ± 2	52 ± 1	158 ± 20	60 ± 2	383 ± 23	82 ± 2	36 ± 7	201 ± 50
3	425 ± 18	109 ± 5	105 ± 57	63 ± 6	507 ± 88	85 ± 1	64 ± 0	267 ± 44
4	957 ± 38	293 ± 16	191 ± 6	156 ± 18	383 ± 4	124 ± 10	53 ± 3	181 ± 12
5	1086 ± 54	288 ± 15	204 ± 22	149 ± 23	442 ± 1	121 ± 10	71 ± 4	161 ± 41
6	1476 ± 69	299 ± 30	191 ± 32	220 ± 8	473 ± 0	100 ± 12	43 ± 2	134 ± 3
7	1531 ± 144	215 ± 51	238 ± 14	192 ± 15	454 ± 43	113 ± 2	56 ± 4	155 ± 26

Results are represented as the mean ± S.D. and expressed per g of dry substrate; Xyl., xylanase; Cel., cellulase; β-gluc., β-glucosidase; Prot., protease; CP, crude protein.

Closely to these results, are the obtained in SSF of run 6 composed by a mixture of 50 % (w/w) of RSC and SBC, particularly for xylanase and protease. As a result of the enzymes production through SSF, the solid substrate composition suffered modifications and the lowest values of cellulose, hemicellulose and lignin content in fermented OCs mixtures was obtained in run 6 with the mixture of RSC and SBC. Despite being observed a lignin concentration in this fermented mixture when compared to the corresponding unfermented mixture (**Table 4.1.**), the fermented conjugation of RSC and SBC showed a lower content of lignin compared to the corresponding fermented single OC's.

Fermented mixture of RSC and SBC also has the highest protein content of all OCs mixtures that is close (93 %) to the obtained with single SBC (OC with highest protein content).

4.3.1.1. Optimum conditions predicted for enzymes production

This experimental design allowed the identification of effects of each OC and their interactions on enzymes production. Additionally, it was possible to find the optimum mixtures of OCs that maximizes the enzymatic activities obtained after SSF. The regression coefficients and statistical parameters of the experimental design are shown in **Table 4.3.** The statistical parameters show a good fit of the models

supported by the coefficient of determination (R^2) and F-value. The coefficient of determination was above 0.96 for all the enzymes except for β -glucosidase (R^2 of 0.74).

Table 4.3. Statistical parameters of simplex centroid mixture design.

RC	Xyl.	Cel.	β -gluc.	Prot.	CP	Cellu- lose	Hemi- cellulose	Lignin
x_1	398.27 ^{***}	53.76 ^{***}	209.31 ^{***}	157.11 ^{***}	396.28 ^{***}	-5.32	-6.42	10.07 ^{**}
x_2	692.02 ^{***}	51.74 ^{***}	158.23 ^{***}	60.08 ^{***}	382.69 ^{***}	-5.30	-6.58 [*]	7.52 ^{**}
x_3	424.79 ^{***}	108.90 ^{***}	104.51 ^{**}	62.95 ^{***}	507.03 ^{***}	-6.54	-10.98 [*]	11.59 ^{***}
x_1x_2	1650.38 ^{***}	962.48 ^{***}	26.98	190.59 [*]	-25.00	19.62	7.11	-13.57
x_1x_3	2698.95 ^{***}	842.60 ^{***}	187.22	129.09 [*]	-37.95	11.56	8.73	-8.81
x_2x_3	3668.46 ^{***}	875.06 ^{***}	239.72	625.01 ^{***}	113.04	3.74	-2.90	-8.95
$x_1x_2x_3$	4203.58 [*]	-4272 ^{***}	815.21	-178.93	534.33	-34.90	-5.21	80.75
Model (SS)	2605850	155247	22027	43198.5	27927.6	39.89	23.85	219.14
Total error (SS)	10051	683.43	7614.89	1605.23	10111.6	19.33	9.42	30.81
R^2	0.996	0.996	0.743	0.964	0.734	0.674	0.717	0.877
R^2 adjusted	0.993	0.992	0.523	0.934	0.506	0.394	0.474	0.771
F-value	302.47	265.02	3.37	31.40	3.22	2.41	2.96	8.30

RC, regression coefficients; R^2 , coefficient of determination; Cel., cellulase; Xyl., xylanase; β -glu., β -glucosidase; Prot., protease; CP, crude protein.

^{***} $P < 0.001$; ^{**} $P < 0.01$; ^{*} $P < 0.05$

These coefficients validate the experimental data, meaning they agree with the predicted ones by the model. Additionally, apart from β -glucosidase, all enzymes showed high F-values indicating that the models were statistically significant ($P < 0.05$). The positive regression coefficients obtained for most of the independent variables represent the synergistic interactions while negative values indicate that the components in the mixture act antagonistically. Despite the type of interaction in each substrate mixture, not all the combinations have a statistically significant effect on the dependent variables' response. Statistically significant effects ($P < 0.001$ and $P < 0.01$) were observed for every independent variable considering cellulase and xylanase production. For β -glucosidase, the mixture of OC had no effects and only the single substrates x_1 , x_2 and x_3 had significant effects ($P < 0.001$ and $P < 0.01$) on this enzyme production. Except for the ternary combination of OC ($x_1x_2x_3$), all the substrate compositions had positive effects on protease production. The coefficients of x_1 , x_2 , x_3 and x_2x_3 had high significant effect ($P < 0.001$) on this enzyme production, followed by x_1x_2 ($P < 0.01$) and finally by x_1x_3 ($P < 0.05$).

The mixture contour plots represented in **Figure 4.1.** show the variation of enzyme activity using different amounts of OC. Each plot represents a dependent variable, and the corner of each triangle represents the OC used as substrates. The surface of the triangle shows changes in substrate composition and the correspondent enzyme production predicted by the model. The darker zone of each triangle represents the optimum mixture of substrates that maximize each dependent variable.

As illustrated in **Figure 4.1.a**, maximum cellulase production was obtained by the binary combinations of OC as discussed above. As shown in **Figure 4.1.b** by the counterplot of xylanase, the darker zone is focused on the center of the triangle but slightly directed to the mixture of RSC and SBC. The mixture of the three OC in equal proportions (run 7) favored the production of b-glucosidase. However, the lower value of the coefficient of determination for this variable, indicates a non-satisfactory model fit. The optimum mixture for protease production was obtained by the combination, in equal proportions, of RSC and SBC. Protease production was increased by 3.7-fold compared to the use of RSC or SBC as single substrates.

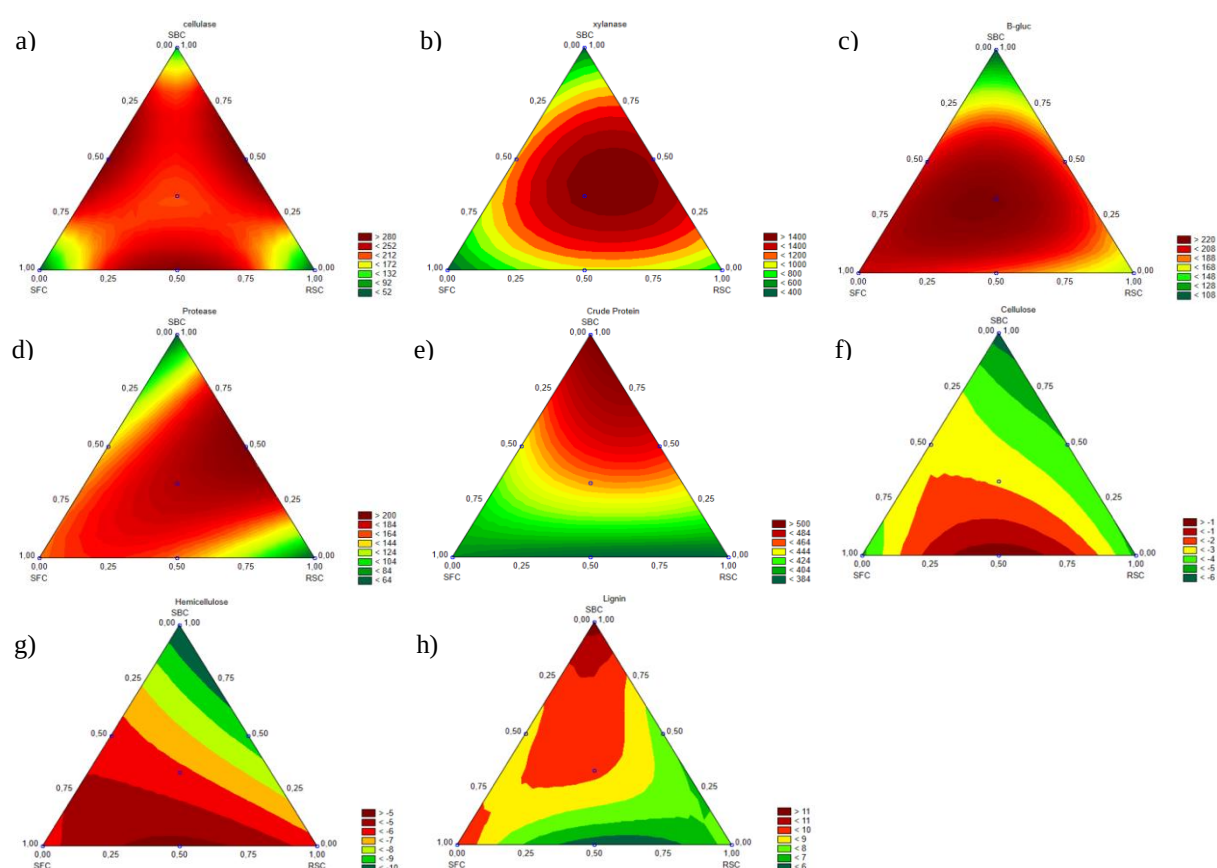


Figure 4.1. Contour plots of each dependent variable obtained by the simplex centroid design after SSF with *A. niger*: a) cellulase; b) xylanase; c) β -glucosidase; d) protease; e) crude protein; f) cellulose; g) hemicellulose; h) lignin.

These results emphasize the importance of substrate optimization for maximization of dependent variables and increase of process yield. The mixture of substrate can overcome the nutritional limitations observed when using single substrates by increasing nutrients availability for the microorganisms.

The synergistic effects of mixing different by-products for the development of an optimized substrate that maximizes enzymatic production has been reported by other authors. Castro et al.²⁷ reported that the combination of agricultural by-products increased the production of protease compared to the use of single substrates in SSF. Additionally, the authors highlighted a medium composed by wheat bran, soybean meal, cottonseed meal and orange peel in equal proportions that showed the strongest synergistic effects of all the combinations, resulting in increased production of protease (11.6 %, 131.4 %, 69.5 % and 547.9 %, respectively for each substrate) after 72 h of fermentation with *A. niger* LBA 02. Leite et al.²⁸ described a higher production of xylanase (710 U g⁻¹) and b-glucosidase (262 U g⁻¹) using a ternary combination composed by crude olive organic pomace, brewer's spent grain and exhausted olive pomace compared to the use of single by-products while maximum cellulase activity (57 U g⁻¹) was obtained in a binary combination of brewer's spent grain and vine trimming shoots. Dias et al.²⁹ identified an optimum mixture composed by the ternary combination of wheat bran, soybean meal and cottonseed meal as an optimum substrate that maximizes the production of L-asparaginase by *A. niger* in SSF.

4.3.1.2. Optimum conditions predicted for nutritional parameters

The chemical composition of OC was evaluated after SSF to understand the effect of enzymes production on lignocellulosic fractions and crude protein (CP). CP was not significantly affected by SSF. OC are natural sources of vegetal protein, and its protein content ranges from 40 to 50 % (w/w) on a dry matter basis, remaining within the same ranges after SSF. On the other hand, hemicellulose and cellulose fractions were strongly affected by SSF. A significant decrease of these fractions was observed in every run of the experimental design. The hemicellulose reduction obtained by SSF of OC mixtures ranged from 47 % (w/w) to 71 %, but cellulose reduction was lower than the observed for hemicellulose, as this component comprises the interior part of the cell wall and presents a crystalline structure. Even though, reductions of 47 % were observed in runs 2 and 3 while the lowest (18 %) was obtained in run 4. After SSF, there was an increased concentration of lignin in every run. The highest value was observed in run 3 representing a lignin concentration nearly 12-fold of the unfermented SBC. The high values of lignin obtained after SSF can be explained by the concentrating effect of SSF as a

consequence of the high reduction of hemicellulose and cellulose. In fact, the absolute lignin mass in the solid, did not significantly change since there was a solubilization of around half of the initial mass of the substrate mostly from the other polysaccharides than lignin. Thus, these soluble compounds were removed during the extraction process leading to a concentration of lignin in the final solid. The coefficients of determination of CP, cellulose, and hemicellulose ranged from 0.67 to 0.73. The statistical parameters obtained for these variables indicate that there is not a good fit by the model to these single dependent variables. As so, no valid conclusions can be outlined regarding the effect of OC mixtures on the crude protein and lignocellulosic composition (cellulose and hemicellulose). On the other hand, for lignin content a coefficient of determination of 0.88 and an F-value of 8.3 were obtained, validating the experimental data fitting to the model. The coefficients of regression indicate that the mixture of OC did not have effects in these dependent variables. Significant effects ($P < 0.001$) of x_1 , x_2 and x_3 were observed for CP and for lignin ($P < 0.001$ and $P < 0.01$). For hemicellulose content only x_2 ($P < 0.05$) and x_3 ($P < 0.001$) showed significant effects. The mixture contour plots indicate that the presence of SBC as substrate in SSF led to the best result in terms of highest CP and the lowest lignin content (**Fig. 4.1.e** and **h**). On the other hand, the absence of SBC in the substrate mixture of SFC and RSC, favored the decrease of cellulose and hemicellulose (**Fig. 4.1. f** and **g**). Other studies evaluated the use of SSF to decrease the fibers content of lignocellulosic materials by other fungi. Xu et al.³⁰ reported a decrease of cellulose (23.5 %), hemicellulose (11.7 %), and lignin (22.4 %) in raw sugarcane bagasse, after 48 h of hydrolysis using a cellulase cocktail obtained after 6 days of SSF of wheat bran with *Inonotus obliquus*. The same author reported decreases of cellulose (18.9 %), hemicellulose (11.2 %), and lignin (14.8 %) for rice straw using the same enzymatic cocktail in saccharification processing. Sousa et al.¹² reported decreases of cellulose, hemicellulose, and lignin after SSF of brewer's spent grain, exhausted olive pomace, exhausted grape marc, and vineshoot trimmings, using independently three different *Aspergillus* species.

4.3.2. Global optimization by multiple response variable

The maximization of multiple variables is a key step to obtain an optimized substrate able to produce a broad range of bioactive compounds with multiple applications via SSF. **Table 4.4.** shows the optimal mixture of OC that maximizes the production of enzymes and CP while minimizing cellulose, hemicellulose, and lignin concentration. The composition of the optimum substrate is comparable to run 6 (**Table 4.1.**) and the predicted values of the dependent variables, by the model are within the range of the experimental values obtained in runs 6 and 7 (**Table 4.2.**).

Table 4.4. Optimization by multiple response variables of enzymes and nutritional properties.

Optimization	Mixture composition (%)		Dependent variables (predicted value): enzymes in U g ⁻¹ , other parameters in g Kg ⁻¹ .							
	RSC	SBC	Xyl.	Cel.	β-glu.	Prot.	CP	Cellulose	Hemi-cellulose	Lignin
Enzymes and nutritional properties	47	53	1466	300	190	217	476	100	40	180

Xyl., xylanase; Cel., cellulase; β-glu., β-glucosidase; Prot., protease; CP, crude protein.

These combinations of OC with optimized chemical composition and simultaneous production of carbohydrases may constitute a value-added product to be applied as animal feed.

4.3.3. Total phenolic compounds and antioxidant potential of optimum substrate mixtures

The effect of substrate composition and SSF on TPC release and antioxidant potential of aqueous extracts of the optimal mixtures of OC was also evaluated (**Fig. 4.2.**).

The concentration of TPC was affected by substrate composition. The presence of SFC in run 7 resulted in a higher initial (control) concentration of TPC compared to run 6. During SSF, the depolymerization of lignocellulosic fractions resulted in the release of phenolic compounds leading to a significantly higher concentration of TPC after SSF, that was significant for both OC mixtures of run 6 and 7, the mixtures with RSC and SBC, and the mixture of the three OC, respectively. The action of enzymes such as β-glucosidase in the release of TPC from cellulosic fractions of fruits and vegetables has been described.³¹ Also, the synergistic action of carbohydrase enzymes on the lignocellulosic matrix of OC exposes lignin, increasing the surface area to the solvent, allowing the extraction of phenolics that were in the form of insoluble-phenolics.³² The presence of phenolic glycosides or phenolics bound to the polysaccharide structure of the cell wall components can be another explanation for the increase of TPC once carbohydrase enzymes can hydrolyze glycosidic bonds releasing phenolic aglycones.³³

As observed in **Fig. 4.2. B-E** the antioxidant potential of aqueous extracts was increased by SSF except for the iron chelation potential (**Fig. 4.2.C**).

The scavenging potential of free radicals (**Fig. 4.2.B**) was increased approximately by 4.6-fold for run 6 while the potential of the ternary mixture was increased by 2.4-fold, compared to respective controls. The scavenging of superoxide radicals (**Fig. 4.2.D**) and extracts reduction potential (**Fig. 4.2.E**) followed the same pattern with increases in the range of approximately 40-fold and 4-fold and 21-fold

and 2.4-fold for runs 6 and 7, respectively. On the other hand, iron chelation potential decreased 2.5-fold and 1.7-fold for run 6 and 7, respectively.

The extracellular enzymes produced during SSF are involved in the release of phenolic compounds bounded to the lignocellulosic matrix of OC. Additionally, these enzymes may alter the chemical structures of phenolic compounds during SSF which would influence the antioxidant potential of samples. The enhanced antioxidant potential can be attributed to the formation of phenolic aglycones as a consequence of deglycosylation by carbohydrases.^{34,35}

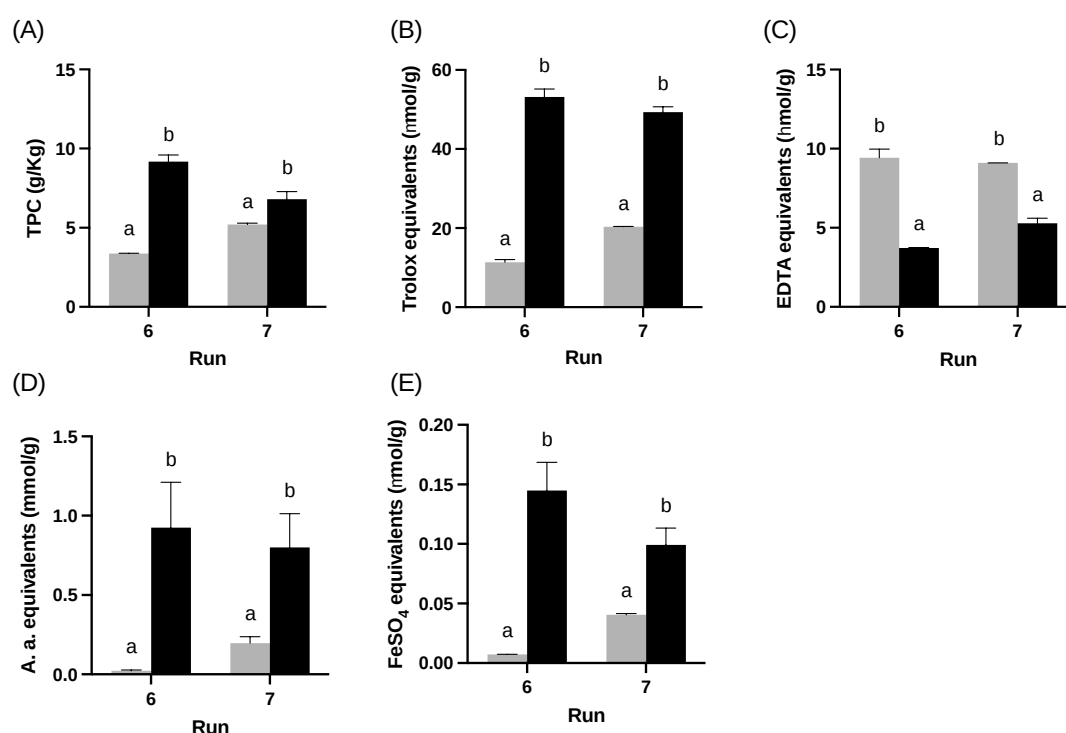


Figure 4.2. Total phenolic content (TPC) and antioxidant potential of aqueous extracts of the optimum mixtures of fermented oilseed cakes (black) with *A. niger*. Control (grey) represents the autoclaved substrates without inoculation and the SSF. (A) TPC; (B) DPPH radical scavenging activity; (C) iron chelation ability; (D) superoxide radical scavenging activity; (E) reducing ability. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters for the same run are not statistically significant different (Tukey test; $P < 0.05$).

Globally, independently of their composition the antioxidant profile of fermented extracts obtained from runs 6 and 7 are quite similar. However, it was observed a higher antioxidant potential in non-fermented extracts from run 7 compared to run 6 resulting in smaller increases of this bioactivity during SSF. This fact may be attributed to substrate composition and the presence of SFC in the ternary mixture of OC. As previously reported by Sousa et al.⁹ SFC shows a higher antioxidant potential compared to RSC and SBC.

4.3.4. Scale-up for tray bioreactor validation

Due to the experimental data similarity observed between runs 6 and 7, these optimum mixtures of OC were selected for scaling-up to tray-type bioreactor. **Table 4.5** depicts the results obtained after the scale-up process for the studied dependent variables. Punctual variations were observed on the dependent variables' responses. Statistically significant differences ($P < 0.05$) were observed in β -glucosidase activity for run 6, considering the use of Erlenmeyer flask and small tray bioreactor. However, considering the same enzymatic activity, experimental results obtained using 1 L Erlenmeyer flasks containing 50 g of substrate were successfully reproduced at a larger scale (400 g tray bioreactor). The antioxidant potential was also evaluated through the scavenging potential of free radicals (DPPH). The results indicate that no statistically significant differences were observed for this bioactivity proving its reproducibility at different scales of the process.

Table 4.5. Scale-up of run 6 using 50 g and 400 g of dry substrate in 1L Erlenmeyer flask and tray bioreactors.

Run	Scale-up	Xyl. (U g ⁻¹)	Cel. (U g ⁻¹)	β -gluc. (U g ⁻¹)	Prot. (U g ⁻¹)	CP (g Kg ⁻¹)	Cellu. (g Kg ⁻¹)	Hemi. (g Kg ⁻¹)	Lig. (g Kg ⁻¹)	DPPH (μ mol TE g ⁻¹)
6	Flask (50 g)	1578 $\pm$ 68	331 $\pm$ 35	207 $\pm$ 27 ^a	146 $\pm$ 18	526 $\pm$ 16	116 $\pm$ 4	41 $\pm$ 0	193 $\pm$ 4	55 $\pm$ 4
	Tray (50 g)	1590 $\pm$ 245	391 $\pm$ 8	299 $\pm$ 47 ^b	169 $\pm$ 11	530 $\pm$ 32	132 $\pm$ 16	59 $\pm$ 7	193 $\pm$ 8	55 $\pm$ 2
	Tray (400 g)	1493 $\pm$ 20	327 $\pm$ 44	139 $\pm$ 20 ^a	180 $\pm$ 3	523 $\pm$ 17	135 $\pm$ 1	56 $\pm$ 4	164 $\pm$ 18	53 $\pm$ 1
7	Flask (50 g)	1259 $\pm$ 263	292 $\pm$ 29	185 $\pm$ 29	176 $\pm$ 11	497 $\pm$ 16	191 $\pm$ 0	78 $\pm$ 2	180 $\pm$ 25	49 $\pm$ 0
	Tray (50 g)	853 $\pm$ 176	254 $\pm$ 29	259 $\pm$ 51	193 $\pm$ 19	481 $\pm$ 69	174 $\pm$ 6	74 $\pm$ 1	186 $\pm$ 20	47 $\pm$ 1
	Tray (400 g)	1270 $\pm$ 54	261 $\pm$ 34	156 $\pm$ 9	179 $\pm$ 3	482 $\pm$ 8	194 $\pm$ 6	81 $\pm$ 3	159 $\pm$ 11	47 $\pm$ 1

Results are represented as the mean $\pm$ S.D. Values with equal letters for each column are not statistically different (Tukey test; $P < 0.05$). Only cases where differences are found are marked; Xyl., xylanase; Cel., cellulase; β -gluc., β -glucosidase; Prot., protease; CP, crude protein; Cellu., cellulose; Hemi., hemicellulose; Lig., Klason lignin; TE, Trolox equivalents.

The same phenomenon regarding antioxidant potential and enzymatic activity was observed for the scale-up of run 7. The crude protein and lignocellulosic composition of fermented OC showed slight variations between experiments during the scale-up process but without statistical significance.

Headspace, that influences the oxygen availability and humidity loss, is one of the main parameters to be considered when optimizing SSF conditions and especially in process scaling-up.¹¹ This parameter may affect the growth and development of microorganisms and consequently the production of bioactive compounds. The headspace ratio between the fermentative bed and the top of the fermenter comparing the 1L Erlenmeyer flask and the tray-type bioreactor was around 3. However, the absence of statistically differences between the fermentative systems clearly indicate that headspace did not influence the SSF process. Also, considering the bed height used in 1L Erlenmeyer flask (2 cm) and in the tray bioreactor (2.5 – 3 cm) the SSF is reproducible.

4.4. Conclusions

OC bioprocessing by SSF, was optimized using a simplex-centroid mixture design that allowed to select the optimum substrate mixture of 50 % (w/w) RSC and 50 % SBC that maximizes carbohydrases and protease production and simultaneously maximize protein content and minimize lignocellulosic components. The mixture of OC resulted in significant increases in cellulase, xylanase, b-glucosidase, and protease production by *A. niger* compared to the single use of OC. The action of lignocellulolytic enzymes in the fibrous fractions of OC was demonstrated, leading to an OC mixture with possible improved digestibility which increases their potential for being used as animal feedstuff. Also, it was successfully demonstrated the potential industrial application of SSF through the scale-up reproducibility of the process.

Additionally, the optimized mixtures under SSF led to increased antioxidant properties, thus boosting the potential application in feed and food industries.

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Chapter 5

Potential of solid-state fermentation to enhance the nutritional value of oilseed cakes for poultry

This chapter was submitted to the Journal of Animal Science and Biotechnology:

Sousa, D.; Moset, V.; López-Luján M. C.; Salgado, J. M.; Dias, A.; Belo, I.; Pascual, J. J.; Cambra-López, M. Potential of solid-state fermentation to enhance the nutritional value of oilseed cakes for poultry

Abstract

The objective of this study was to evaluate the potential of solid-state fermentation (SSF) to enhance the nutritional value of oilseed cakes (OC) for poultry. To this end, firstly, we characterized the main nutrients and antinutritional factors in non-fermented and fermented OC, as well as in fermented OC submitted to an extraction process from where enzymatic extracts were recovered. Secondly, we assessed the *in vitro* digestibility of dry matter (DM), organic matter (OM) and crude protein (CP) from these products using a poultry *in vitro* digestion model. Additionally, raw OC were supplemented with enzymatic concentrated extracts generated during SSF and their effect on nutrient digestibility was also evaluated.

SSF efficiently increased the nutritional value of OC (24 % of CP, 6 % of DM and 39 % of minerals) and was able to decrease fiber fractions and antinutritional factors. This bioprocess, however, compromised the protein quality and its bioavailability which resulted in lower amino acids concentration, lower soluble protein, lower available lysine, and higher protein dispersibility. This was attributed to a transformation of true protein into nonprotein compounds such as ammoniacal nitrogen. The aqueous extractions led to the obtainment of a concentrated protein fraction with highly undigestible fiber. Solid-state fermented mixtures significantly increased the DM, OM, and CP *in vitro* digestibility coefficients compared with non-fermented. Enzymatic supplementation did not statistically modify digestibility parameters.

This study demonstrated that SSF is an effective approach for the obtainment of value-added products, but special care should be taken to the structural properties of proteins specially if applied for animal feed.

Keywords: *in vitro*; poultry; oilseed cakes; solid-state fermentation; enzyme supplementation; digestibility.

5.1. Introduction

Global consumption of animal proteins according to OECD-FAO Agricultural Outlook predictions, are expected to increase by 14 % by 2030 compared to the period of 2018-2020.¹ Over the last years, poultry is becoming the consumers' preference choice of animal protein supply,¹ due to the lower price of chicken compared to other meats, its healthy fatty acid profile and its convenience of preparation. In fact, this protein source is expected to represent 41 % of all protein from meat sources by 2030.¹

Maize and soybean are the most common energy and protein sources in poultry feed worldwide, respectively.² Undoubtedly soybean cake is the highest produced oilseed cake worldwide, followed by rapeseed cake and sunflower cake.¹ However, the fibre content of these products, particularly the amount of protein bonded in the fibre matrix (i.e. neutral detergent fibre protein), as well as the presence of antinutritional factors may reduce the availability of certain nutrients and thus limit their nutritional value when used as animal feed.³

Due to the projected growth of the poultry industry, it is essential to find alternative sources of protein for feed that can reduce the pressure on natural resources and/or implement feed valorisation treatments that can enhance the nutritional value of the feed while maximizing feed efficiency and optimizing resources use. Indeed, this would contribute to moving animal feeding systems towards sustainable intensification premises; increasing production yields in ways that place far less pressure on the environment and without compromising the capacity to continue producing food in the future.⁴

Solid-state fermentation (SSF) can be used to valorise agro-industrial by-products using an eco-friendly approach with low environmental impact.⁵⁻⁸ Through SSF, microorganisms grow on the surface of solid materials. This process occurs in the near absence of free water mimicking the conditions of the natural habitat and filamentous fungi are well-adapted microorganisms to this type of fermentation. Filamentous fungi can produce extracellular enzymes that can hydrolyse the lignocellulosic structure of cell wall polymers present in plants, decreasing the low-nutritional fibre content of these materials. Lignocellulolytic enzymes such as cellulase and xylanase can further degrade cellulose and hemicellulose polysaccharides into simple oligosaccharides chains, making fibre-associated proteins more accessible. Additionally, the deconstruction of the lignocellulosic network may allow the release of phenolic compounds.⁹ However, despite the recognized advantages of SSF to improve physicochemical properties of by-products and agro-food wastes, few studies have explored their potential to enhance the nutritional value of these vegetable products for animal feeding.

Non-invasive *in vitro* models, that can mimic the physiological and chemical characteristics of animals' gastrointestinal tract, can be used in the laboratory to evaluate the nutritive value of feedstuffs. Ideally,

these models should be reproducible, cheaper than *in vivo* experiments and simple to perform giving fast results at the same time.¹⁰ Moreover, they can contribute to implementing the three Rs (Replacement, Reduction and Refinement) in animal research.¹¹ *In vitro* digestibility methods can be employed to evaluate the digestibility of feed ingredients, the efficiency of exogenous enzymes and nutrient availability in a diet.¹²

In this framework, our goal was to evaluate the potential of SSF to enhance the nutritional value of oilseed cakes (OC) for poultry. To this end, firstly, we characterized the main nutrients and antinutritional factors in non-fermented and fermented OC, as well as in fermented OC submitted to an extraction process from where enzymatic extracts were recovered. Secondly, we assessed the *in vitro* digestibility of dry matter (DM), organic matter (OM) and crude protein (CP) from these products using a poultry *in vitro* digestion model. Additionally, raw OC were supplemented with enzymatic concentrated extracts generated during SSF and their effect on nutrient digestibility was also evaluated.

5.2. Materials and methods

5.2.1. Oilseed cakes and solid-state fermentation process

Three OC from the vegetable oils industry were used: sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC). The SFC was provided by Sorgal SA, while RSC and SBC were provided by IBEROL (Sociedade Ibérica de Oleaginosas, SARL) both in Portugal. Following previous optimization studies reported by Sousa et al.¹³ OC were blended in different proportions and identified as mixture 1, NF1 (non-fermented 1), representing the combination of 1:1 (w/w) RSC:SBC, and mixture 2, NF2 (non-fermented 2), being the combination of the three OC in a ratio of 1:1:1.

The NF1 and NF2 were subjected to SSF in tray type bioreactors, as previously described by Sousa et al.¹³ using *Aspergillus niger* CECT 2915 strain. Briefly, a total amount of 400 g of dry OC mixtures was used with moisture adjusted to 75 % with distilled water (w/w, wet basis). OC were sterilized for 15 min at 121 °C. A sterile solution of peptone composed of 1 g L⁻¹ and 0.1 g L⁻¹ Tween-80 was used to recover *A. niger* spores from PDA slants and to prepare an inoculum suspension with an adjusted concentration of 10⁶ spores mL⁻¹, from which 80 mL was added to each tray. After inoculation trays were kept on a controlled temperature chamber for 7 days at 25 °C. Each experiment was performed in duplicate and a control experiment without inoculation was performed in the described conditions.

After SSF, three different fractions were obtained and identified as i) fermented (F), representing the whole fermented residue obtained after SSF; ii) fermented-extracted (F_{EXT}), representing the fermented residue obtained after SSF and extracted with distilled water in a ratio of 1:5 (w/w) solid: liquid; and iii)

liquid fermented (LF), liquid fraction obtained from the extraction of each combination of residues containing the enzymatic concentrated extracts. Therefore, three samples were obtained from each mixture (Mix1 and Mix2): non-fermented (NF), F and F_{EXT}; resulting in six samples in total; that were subsequently used in the *in vitro* tests.

Additionally, to evaluate the effect of supplementation with enzymatic concentrated extracts from SSF, raw (non-fermented) SFC, RSC, and SBC, supplemented with LF extract were also subjected to *in vitro* assay.

The LF extracts contained carbohydrase's (cellulase, xylanase and β -glucosidase) and protease enzymatic activity (previously characterized by Sousa et al.¹³). Due to the similarity between LF extracts from Mix1 and Mix2, LF1 (LF from Mix1) was selected to be used in this work. **Table 5.1.** shows the enzymatic characterization of LF1.

Table 5.1. Enzymatic characterization of liquid concentrated extract from fermentation of mixture 1 (LF1).

Enzyme	Activity (U g ⁻¹)
Cellulase	327 $\pm$ 44
Xylanase	1493 $\pm$ 20
β -glucosidase	139 $\pm$ 20
Protease	180 $\pm$ 3
Phytase	78 $\pm$ 11

Three enzymatic concentrations were evaluated, corresponding to: level 1, 0 Units per kg of raw substrate; level 2, 2194 U of cellulase, 10018 U of xylanase, 932 U of β -glucosidase, 1207 U of protease and 500 U of phytase per kg of raw substrate and level 3, 4388 U of cellulase, 20036 U of xylanase, 1864 U of β -glucosidase, 2414 U of protease and 1000 Units of phytase per kg of raw substrate. To achieve the final concentrations of level 2, 1.342 g of lyophilized enzymatic cocktail was dissolved in 50 mL of 0.25 M acetate buffer, pH 5.5 and 125 μ L of the mixture was added to the reaction mixture. To achieve the final concentration of level 3, 250 μ L of enzymatic extract was added to the reaction mixture. All samples were freeze-dried and stored at -20 °C before analyses.

Figure 5.1. shows a detailed scheme of the experimental procedure used in this work.

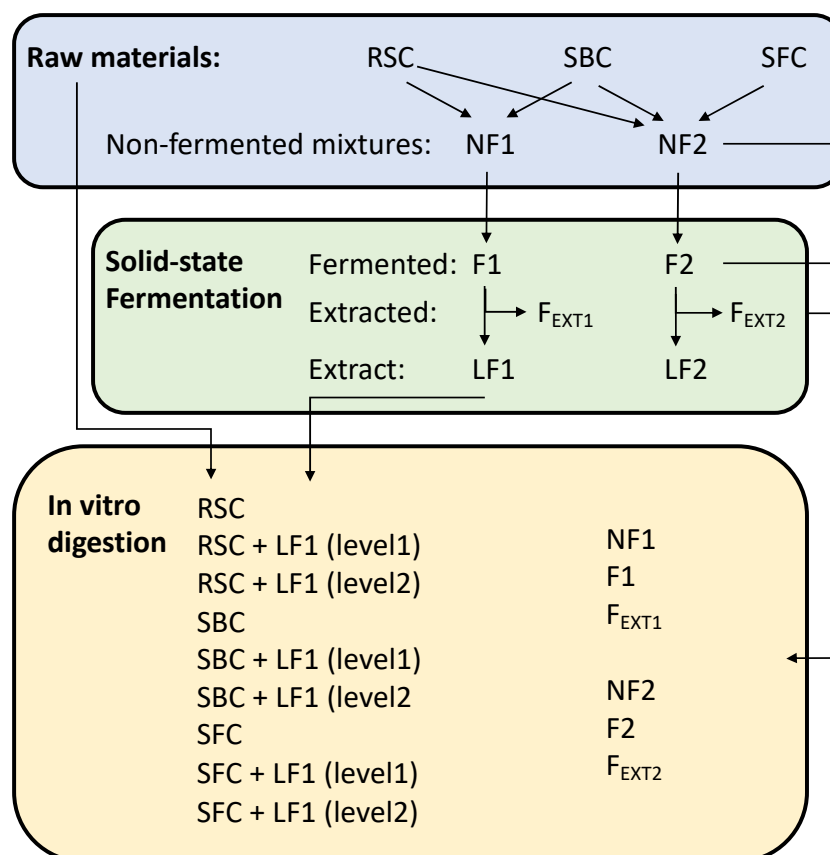


Figure 5.1. Experimental design scheme. RSC: rapeseed cake; SBC: soybean cake; SFC: sunflower cake. NF: no-fermented, F: fermented; FEXT: fermented and extracted; LF: liquid fermented. Level 1: 500 U phytase per kg of raw material; Level 2: 1000 U phytase per kg of raw material.

5.2.2. Enzymatic activity of LF extracts

The enzymatic activity of cellulase, xylanase, β -glucosidase and protease were quantified as previously described by Sousa et al.¹³ One unit of enzyme activity was defined as the amount of enzyme required to release 1 μ mol of glucose or xylose, respectively, for cellulase or xylanase, per minute at 50 °C and pH 4.8. One unit of β -glucosidase enzyme activity was defined as the amount of enzyme required to release 1 μ mol of *p*-nitrophenol per minute at 50 °C and pH 4.8. One unit of protease enzyme activity was defined as the amount of enzyme required to release 1 μ mol of azopeptides in 1 min at 37 °C and pH 5.0.

Phytase activity was determined following the method described by Gunashree and Venkateswaran.¹⁴ One unit of phytase activity was defined as the amount of enzyme required to release 1 μ mol of inorganic phosphorus from the substrate in 1 min at 40 °C.

5.2.3. Nutrient composition

The NF, F and F_{EXT} samples from mixtures 1 and 2 were analysed in duplicate using methods of the AOAC,¹⁵ for DM (934.01) and ashes (942.05). The nitrogen content was quantified using the Kjeldhal method,¹⁶ and estimation of crude protein was obtained using a defined factor of 6.25. The neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) fractions were analysed sequentially according to Mertens et al.¹⁷ procedure 973.18 of the AOAC,¹⁵ and Van Soest et al.¹⁸, respectively, with a thermo-stable α -amylase pre-treatment and expressed exclusive of residual ash, by using a nylon filter bag system (Ankom, Macedon, NY, USA). Amino acids profile was quantified using Waters High-Performance Liquid Chromatography (HPLC) with a column Waters Acc. Tag (3.9 d.i. x 150 mm). Prior to HPLC analysis, for total amino acids quantification, samples were first subjected to acid hydrolysis with HCl at 110 °C for 23 h. For quantification of sulphurated amino acids, samples were primarily oxidized with performic acid for 16 h at 4 °C, followed by acid hydrolysis with HCl at 110 °C for 23 h.

True protein content was calculated based on the difference between total nitrogen determined by Kjeldhal and non-protein nitrogen (urea and ammoniacal nitrogen). To obtain the value of the true protein content, the amount of nitrogen obtained by this difference was multiplied by a factor of 6.25.

5.2.4. Antinutritional factors and protein quality parameters

The presence and quantification of antinutritional factors was performed in each sample of NF, F and F_{EXT} from mixture 1 and 2. Phytic acid content was determined with the phytic acid/total phosphorus K-PHYT (Megazyme International, Ireland) enzymatic kit. Tannins were quantified through ultraviolet-visible (UV/VIS) spectrophotometry. Glucosinolates were quantified by volumetric analysis through the determination of allyl isothiocyanate by steam distillation based on BOE.¹⁹ The presence of erucic acid was quantified using gas chromatography. Protein solubility in potassium hydroxide (KOH) was determined following the method described in Araba & Dale,²⁰ protein dispersibility index (PDI) and trypsin inhibitor was determined following AOCS,²¹ and urease activity index was determined using a modified method of Caskey & Knapp,²² based on pH increments. Determination of available lysine was performed through UV/VIS spectrophotometry.

5.2.5. *In vitro* poultry digestion model

To evaluate the *in vitro* digestibility coefficients of NF, F and F_{EXT} from SSF process (of both mixtures), as well as the raw materials supplemented with LF1 concentrated extract (at 3 levels), fifteen samples were analysed in triplicate using an *in vitro* digestion model for monogastrics.^{23,24} The *in vitro* DM, OM, and CP digestibility coefficients were determined. DM, ashes and CP of the residue after digestion were analysed using methods of the AOAC,¹⁵ as previously described.

The *in vitro* procedure used was a two-stage sequential protocol where the first stage was the gastric phase (proventriculus and gizzard) digestion using pepsin in the presence of hydrochloric acid (HCl) and the second stage the simulation of small intestinal digestion using pancreatin solution, following Farhadi et al.²⁵ Prior to the gastric phase, the crop phase was simulated as described in Hirvonen et al.²⁶ Briefly, to simulate the crop phase, 250 mg CP ($N \times 6.25$) of each sample was introduced in 50 mL Erlenmeyer flasks and 3 mL of phosphate-free sterile water was added. Samples were kept at 40 °C for 20 min. After incubation, 4.5 mL of freshly prepared pepsin from porcine gastric mucosa, containing 2,000 U of pepsin per mL (P6887, Sigma-Aldrich) dissolved in 0.13 M HCl, was added to each flask. The pH was adjusted to 2.5 with 6 M HCl or 6 M NaOH. Flasks were placed on a shaking water bath at 140 strokes per minute for 42 min at 40 °C. At the end of this stage (gastric phase), 1.5 mL of 1M NaHCO_3 containing 3 mg mL^{-1} of pancreatic solution from porcine pancreas (P7545, Sigma-Aldrich) was added to each flask. Flasks were vortexed and pH was adjusted to 6.3 to 6.7 with 6 M HCl or 6 M NaOH. All flasks were placed on the shaking water bath for 60 min at 40 °C. At the end of the small intestine phase, 1 mL of 100 g L^{-1} trichloroacetic acid was added to each tube. Tubes were kept at 20 °C for 30 min. Precipitated protein fractions were filtered by a Gooch crucible (pore diameter: 40 to 90 μm) in a cold extraction unit of fiber 1021 cold extractor, Tecator™, Denmark. Sediments were rinsed three times sequentially with water, ethanol, and acetone according to Kovitvadhi et al.²⁷ Celite was used during the filtration process. Crucibles were dried at 105 °C for 24 h and recovered samples were stored at 20 °C until analysis.

5.2.6. Calculations and statistical analysis

Digestibility of DM, OM and CP in percentage were calculated as the difference between the initial nutrient content of each sample and the undigested nutrient in the residue, corrected for the blank, with respect to the initial nutrient content and multiplied by 100.

Analysis of variance for *in vitro* digestibility and phytase activity followed by post hoc Turkey's honestly significant difference (HSD) test was carried out using Statistical Analysis System (SAS) software. Data

from NF, F and F_{EXT} were analysed using a model that included the treatment, the mixture, and their interaction as fixed effects. Data from raw materials with LF were analysed using a model that included the raw material, the LF level and their interaction as fixed effects.

5.3. Results

5.3.1. Effect of solid-state fermentation on oilseed cakes characterization (nutrient composition and antinutritional factors)

5.3.1.1. Nutrient composition

Table 5.2. shows the chemical composition of the OC used in this study, as well as the mixtures created, before the SSF (NF), after the SSF (F) and after extraction of the LF extracts (F_{EXT}).

The composition of single OC and NF mixtures of OC obtained showed large variations in CP from 41 to 50 % DM, and in fibrous fractions (NDF, varying from 17.2 to 48.1 %; ADF, varying from 10.3 to 31.3 % and ADL, varying from 0.14 to 11.2 % DM). SBC had the highest CP and the lowest fiber concentrations, while RSC showed the highest concentration of fibrous fractions.

Compared to NF mixtures, F mixtures exhibited an increase in DM (1.06-fold), CP (1.24-fold), ash (1.39-fold) and ADL (1.3-fold for F_1) contents.

The degradation of hemicellulose and cellulosic fractions of the OC by the microorganisms during SSF led to an increase in lignin concentration (ADL fraction).

As regards the fermented OC obtained after the extraction of soluble compounds (F_{EXT}), these were characterized by a high concentration of fibers fractions, as soluble fractions were recovered in the LF extract which contained a concentrated cocktail of enzymes with soluble minerals, sugars, proteins, and phenolic compounds. An increase of ash and thus of the mineral portion of OC could be observed after SSF.

Table 5.2. Nutrient composition of non-fermented (NF), fermented (F) and fermented-extracted (FEXT) oilseed cake mixtures from sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC) on dry matter basis (% DM).

% g/100g DM	Raw Oilseed cakes			Mixture 1, ratio 1:1 (RSC:SBC)			Mixture 2, ratio 1:1:1 (SFC:RSC:SBC)		
	SFC	RSC	SBC	NF1	F1	F _{EXT} 1	NF2	F2	F _{EXT} 2
DM	89.93	85.86	89.84	87.21	92.86	94.28	87.92	93.13	93.83
Ash	9.10	8.73	7.66	7.90	10.88	8.19	8.09	11.31	8.04
Crude protein	45.75	43.82	49.12	44.47	55.80	50.29	42.96	56.05	46.15
Neutral detergent fiber	40.73	48.11	17.22	32.23	23.78	31.16	35.76	25.09	34.57
Acid detergent fiber	27.51	31.26	10.26	20.51	16.83	26.24	24.22	18.63	25.38
Acid detergent lignin	6.87	11.19	0.14	5.70	7.41	11.14	7.67	7.65	10.52
True protein	40.5	38.6	44.1	39.7	33.3	38.0	38.6	31.7	34.9
Ammoniacal N	0.06	< 0.05	< 0.05	< 0.05	2.68	1.18	0.05	2.93	1.07
Amino acids									
Asp	4.49	3.07	7.66	4.78	3.96	3.48	4.78	3.96	4.19
Ser	2.24	1.83	4.93	2.38	1.95	1.78	2.38	2.00	2.20
Glut	9.32	7.67	10.39	9.29	6.87	5.36	8.98	4.92	5.68
Gly	3.08	2.69	3.03	2.91	2.48	2.31	2.53	2.12	2.51
His	1.27	1.44	2.10	1.56	0.59	0.98	1.16	0.87	1.12
Arg	3.83	2.52	4.32	3.53	2.80	2.28	3.18	2.05	2.24
Thr	1.73	1.86	2.60	2.14	1.94	1.69	1.81	1.54	1.70
Ala	1.84	1.86	2.64	2.11	2.04	1.98	1.90	1.67	2.02
Pro	1.81	2.57	2.95	2.66	1.99	1.82	2.21	1.61	1.79
Cys	0.51	0.80	0.69	0.80	1.03	0.50	0.42	0.67	0.69
Tyr	1.10	1.29	2.26	1.64	1.64	1.26	1.21	1.12	1.19
Val	2.64	2.66	3.51	2.88	2.50	2.50	2.44	2.19	2.59
Met	1.13	1.13	0.98	1.06	1.02	1.09	1.09	1.01	1.19
Lys	1.70	2.22	3.70	2.88	2.04	1.94	2.28	1.68	1.93
Ile	1.99	1.89	3.09	2.34	1.84	1.90	1.97	1.60	1.91
Leu	3.13	3.32	4.83	4.00	3.43	3.20	3.31	2.58	3.22
Phe	1.98	1.67	2.85	2.24	1.84	1.69	1.88	1.39	1.69

D. M.– dry matter, C- carbon, N- nitrogen, C. P.– crude protein, NDF- neutral detergent fiber, ADF- acid detergent fiber, ADL- Acid detergent lignin, Asp- Aspartic acid, Ser- Serine, Glut- Glutamic acid, Gly- Glycine, His- Histidine, Arg- Arginine, Thr- Threonine, Ala- Alanine, Pro- Proline, Cys- Cysteine, Tyr- Tyrosine, Val- Valine, Met- Methionine, Lys- Lysine, Ile- Isoleucine, Leu- Leucine, Phe- Phenylalanine

According to **Table 5.2.**, the aminoacidic profile of OC and its mixtures was affected during SSF. Decreases in almost all amino acids could be observed after SSF. Cysteine was the only amino acid's concentration that increased during SSF. A 1.29-fold (mixture 1) and 1.60-fold (mixture 2) increase of this amino acid was observed for F. Methionine concentration was slightly affected by SSF with reductions of 3.77 % (mixture 1) and 7.34 % (mixture 2) in F.

After SSF there was a decrease of true protein values in comparison to NF mixtures and a simultaneously increase of ammoniacal nitrogen. True protein reductions of 1.19 and 1.22-fold could be observed between NF mixtures and F1 and F2, respectively. On the contrary, the ammoniacal nitrogen concentration increase 54 and 59-fold for F1 and F2, compared to the respective NF mixtures.

5.3.1.2. Antinutritional factors and protein quality

Table 5.3. shows the characterization of the main antinutritional factors and protein quality parameters present in the OC in study. A 11.4-fold decrease in phytic acid (mixture 1) and 8.9-fold (mixture 2) was observed for F; and a 12.4-fold decrease in phytic acid (mixture 1) and 9.3-fold (mixture 2) for F_{EXT} compared with NF.

Table 5.3. Antinutritional factors and protein quality parameters of non-fermented, fermented and fermented-extracted oilseed cake mixtures from sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC) on dry matter basis.

	NF1	F1	F _{EXT} 1	NF2	F2	F _{EXT} 2
Antinutritional factors (% w/w)						
Phytic acid	2.97	n.d.	0.24	3.55	n.d.	0.38
Tannins	0.03	0.06	0.03	0.03	0.06	0.05
Glucosinolates	0.01	< 0.001	< 0.001	0.01	0.04	0.04
Erucic acid ¹	0.15	0.29	0.34	0.10	0.20	0.25
Physical chemical protein properties						
Soluble Protein (KOH) (%)	60.89	53.31	54.62	64.71	55.84	53.61
Protein dispersibility index (%)	15.31	26.49	17.29	23.57	29.53	20.89
Trypsin inhibitor (mg/g)	0.08	0.17	0.11	0.05	0.30	0.10
Urease Activity Index ²	0.18	0.02	0.04	0.17	0.01	0.05
Available Lysine (%)	2.01	1.86	1.73	1.76	1.42	1.50

n.d., non-determined; ¹% of erucic acid expressed in 100% of fatty acids; ²Units change in pH using the modified Caskey-Knapp procedure

Tannins concentration increased with SSF and in F mixtures, the concentration of these compounds doubled compared to NF. The concentration of these compounds was lower in F_{EXT}. Glucosinolates

content was reduced during SSF in F and F_{EXT} when compared to non-fermented in mixture 1; but increased in F and F_{EXT} , when compared to non-fermented in mixture 2.

The concentration of erucic acid was affected during SSF showing increases that ranged from 1.9 to 2.5-fold in both mixtures.

Decreases ranging from 12 to 17 % of protein solubility in KOH were observed after SSF. On the other hand, an increment of protein dispersibility index (PDI) was observed for the fermented mixtures of OC. The protein dispersibility index of F mixtures increased 42 % (mixture 1) and 20 % (mixture 2) compared with non-fermented mixtures. A smaller increment of 11 % was observed for F_{EXT} in mixture 1. The PDI of F_{EXT} in mixture 2 decreased 11 % comparing to non-fermented mixture 2.

The concentrations of trypsin inhibitors present in SFC, and RSC was negligible. Despite showing a higher concentration of this factor compared to other OC, in our study, SBC showed a relatively low concentration of trypsin inhibitors. The aqueous extraction after SSF resulted in fermented extracts (F_{EXT}) with lower content of trypsin inhibitors. Compared to F mixtures, a 1.5 and 3-fold reduction was observed for F_{EXT} (mixture 1) and F_{EXT} (mixture 2).

The results of the urease activity index reported decreases after SSF resulting in a lower activity of this enzyme. A 9 and 17-fold difference could be observed comparing F mixture 1 and 2, respectively compared with the non-fermented mixtures. The results of F_{EXT} mixtures also showed lower values of urease index when compared to NF being possible to observe a 4.5 and 3.4-fold reduction of this index compared to the respective NF mixtures.

Reductions of lysine content ranging from 7 to 15 % were observed after SSF for both mixtures of OC.

5.3.2. Effect of solid-state fermentation of oilseed cakes on in vitro nutrient digestibility

Table 5.4. depicts the results of DM, OM and protein digestibility of NF, F and F_{EXT} of OC mixtures 1 and 2. There was a significant effect of the mixture and treatment on digestibility coefficients of DM, OM, and CP, except for the mixture effect for CP digestibility. In addition, there was a significant effect of the interaction between mixture and treatment on DM and OM digestibility.

Average *in vitro* digestibility coefficients for DM for NF and F products ranged from 44 to 54 %, while F_{EXT} coefficients were around 33 to 36 %. The OM digestibility coefficients varied from 71 to 86 % for all products. The crude protein digestibility coefficients for NF and F mixtures differed from 62 to 70 % while for F_{EXT} they were around 48 to 50 %.

Table 5.4. In vitro nutrient digestibility coefficients (dry matter-DM dig, organic matter-OM dig and crude protein- CPdig) of non-fermented, fermented and fermented-extracted oilseed cake mixtures from sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC).

	DMdig (%)	OMdig (%)	CPdig (%)
NF1	49.82 ^b	81.37 ^b	64.35 ^{bc}
F1	53.59 ^a	85.98 ^a	67.51 ^{ab}
F _{EXT} 1	35.27 ^d	72.67 ^d	49.47 ^d
NF2	44.78 ^c	77.17 ^c	62.91 ^c
F2	52.16 ^{ab}	85.67 ^a	69.58 ^a
F _{EXT} 2	33.11 ^d	71.60 ^d	48.01 ^d
SEM	0.737	0.736	0.865
P-value treatment x mixture	0.0294	0.0012	0.0838
P-value treatment (NF, F, F _{EXT})	<0.0001	<0.0001	<0.0001
P-value mixture (1,2)	<0.0001	0.0004	0.7028

DM, dry matter; OM, organic matter; NF, non-fermented; F, fermented; F_{EXT}, fermented extracted; SEM, standard error of the mean. Results represent the average of five independent batches. Values with equal letters are not statistically significant different (Tukey test; $P < 0.05$). Statistical analysis is related to each digestibility parameter.

The DM and OM *in vitro* digestibility coefficients were significantly ($P < 0.05$) higher in F mixtures compared to NF and F_{EXT}, following the same pattern for both OC mixtures. Higher increases of DM and OM coefficients were observed for the ternary combination of OC (mixture 2, composed of 1:1:1 ratio of all three OC).

5.3.3. Effect of supplementation with enzymatic concentrated extracts generated during solid-state fermentation, on in vitro nutrient digestibility coefficients of oilseed cakes

Table 5.5 depicts the results of the *in vitro* digestion coefficients of NF OC with different concentrations of enzymatic supplementation.

Results show the supplementation with enzymes did not significantly affect *in vitro* nutrient digestibility coefficients in our work.

Table 5.5. Dry matter digestibility, organic matter digestibility and protein digestibility of non-fermented oilseed cakes with three levels of external enzyme supplementation.

	DMdig (%)	OMdig (%)	CPdig (%)
SFC + no enzyme	44.03 ^b	76.44 ^b	71.98 ^a
SFC + enzyme level 1	42.96 ^b	76.14 ^b	68.00 ^a
SFC + enzyme level 2	44.50 ^b	77.06 ^b	68.02 ^a
RSC + no enzyme	32.67 ^c	66.53 ^c	42.98 ^b
RSC + enzyme level 1	32.49 ^c	65.51 ^c	41.60 ^b
RSC + enzyme level 2	31.77 ^c	66.06 ^c	40.23 ^b
SBC + no enzyme	62.00 ^a	90.32 ^a	72.86 ^a
SBC + enzyme level 1	61.56 ^a	89.60 ^a	73.14 ^a
SBC + enzyme level 2	64.60 ^a	91.60 ^a	76.83 ^a
SEM	1.418	0.825	1.985
P-value substrate x enzyme level	0.5440	0.2394	0.3079
P-value substrate	<0.0001	<0.0001	<0.0001
P-value enzyme level	0.6837	0.8328	0.5848

DM, dry matter; OM, organic matter; SFC, sunflower cake; RSC, rapeseed cake; SBC, soybean cake; enzyme level 1, supplementation with 2194 U of cellulase, 10,018 U of xylanase, 932 U of β -glucosidase, 1,207 U of protease and 500 U of phytase per kg of raw substrate; enzyme level 2, supplementation with 4388 U of cellulase, 20,036 U of xylanase, 1,864 U of β -glucosidase, 2,414 U of protease and 1000 Units of phytase per kg of raw substrate; SEM, standard error of the mean. Results represent the average of six independent experiments. Values with equal letters are not statistically significant different (Tukey test; $P < 0.05$). Statistical analysis is related to each digestibility parameter.

5.4. Discussion

The nutritional properties of single OC (SFC, RSC and SBC) used during this study were within the standard parameters recommended for animal feed formulations according to INRAE et al.²⁸ and close to that previously reported by Sousa et al.⁶ for these same raw materials.

The SSF modified OC nutrient composition and these changes could be attributable to the inherent fungi hydrolytic process. The production of microbial protein, generally referred to as single cell protein during SSF by *Aspergillus niger* is one of the factors that may have contributed to the increase of CP.

^{5,29,30} During SSF, however, the production and release of proteinaceous compounds such as enzymes

and the production of nonprotein compounds including: ammonia, amino sugars such as glutamine and chitin, formation of complexes such as phenol-protein, protein-lignin or protein-chitin can also occur.^{31,32} Also, during fermentation microorganisms use carbohydrates as carbon source to support their growth affecting the C/N ratio leading to a fermented product with concentrated nitrogen resulting in higher levels of protein in the total mass.³³ The concentration effect of ADL observed after SSF is the result of the degradation of fibrous compounds namely cellulose and hemicellulose present in OC matrix with the exception of lignin which is the most undigestible fraction and recalcitrant to degradation. The higher concentrations of minerals observed after SSF can be attributed to the metabolic activity of the microorganism during the fermentation process or also to the decrease of fibres fraction.

The amino acid profile of OC is a key relevant variable for determining its nutritional value when it is intended to be used in animal feed. The obtained results indicate a clear decrease of amino acids after SSF. Several amino acids are involved in enzymatic reactions and protein synthesis and may have contributed to this decrease. Additionally, the conversion of true protein into ammoniacal nitrogen could partly explain these differences. During SSF, transformation of true protein into nonprotein compounds such as ammoniacal nitrogen has been reported.³⁴ With the aqueous extraction, soluble compounds of the lignocellulosic matrix of OC as well as nonprotein compounds were eluted resulting in a concentrated F_{EXT} with lower levels of ammoniacal nitrogen and higher levels of true protein in comparison to F.

The presence of antinutritional factor's in OC is one of the main drawbacks that limit their nutritional value as animal feed.³⁵ Besides antinutritional factors, physical-chemical protein properties (e.g., protein solubility, urease activity and reactive lysine, amongst others) are valuable indicators of the protein quality and availability of the amino acids contained in them for the animals.³⁶ Phosphorus (P) is an essential and limiting nutrient in poultry feed and phytic acid or phytate is the main storage form of organic P in plant seeds. As poultry do not generate sufficient phytase that can hydrolyse phytic acid, the P contained in the phytic acid molecule cannot be utilized by the animals.³⁷ Additionally, phytic acid is a strong chelation agent that binds to important mineral cofactors influencing the activity of digestive proteins.³⁸ The potential of SSF to decrease phytic acid concentrations has been previously reported,^{39,40} and can be attributed to the production of the extracellular enzyme phytase by *A. niger*.

Tannins are naturally occurring groups of phenolic compounds, considerable undesirable in animal nutrition as they precipitate proteins, may inhibit digestive enzymes, limits minerals and vitamins

utilization and can negatively affect feed palatability.⁴¹ However, the presence of small concentrations of tannins has been reported to improve the overall health status, nutrition, and animal performance in monogastrics.^{42,43} These positive effects are related to the promotion of intestinal ecosystem health through tannins anti-oxidant, anti-microbial and anti-inflammatory potential.⁴⁴ The increment of tannins during SSF may be related to the delignification of lignocellulosic fractions of OC. Bound phenolic compounds can be connected to structures of vegetable cell walls such as hemicellulose or lignin and can be released by the action of extracellular enzymes produced during SSF. The release of phenolic compounds from the OC matrix, using the same OC has been previously described by Sousa et al.⁶ Additionally, tannins are water soluble compounds and for that reason their concentration decreased after SSF in F_{EXT}.

Glucosinolates and erucic acid are characteristic antinutritional factors of RSC. The main toxic compounds are glucosinolates and their degradation products. High glucosinolates ingestion in hens increased mortality and lowered egg production as well as egg weights.⁴⁵ On the other hand, the main effects observed when feeding poultry with diets containing high erucic acid were growth retardation and cardiac lipidosis.⁴⁶ The observed decrease of these compounds in mixture 1 may be related to the capacity of *A. niger* to produce a myriad of extracellular enzymes able to degrade these compounds.⁴⁷ On the other hand, despite the increase of erucic acid after SSF, the concentration of this parameter in these OC was considerably low representing less than 0.5 % of total fatty acids. According to the European Food Safety Authority, the lowest level with adverse observed effects identified for liver toxicity in poultry was after exposure to erucic acid in a concentration of 20 mg Kg⁻¹ by weight, per day.⁴⁶

As regards the physical-chemical properties of proteins, protein solubility in KOH and protein dispersibility index (means of comparing the solubility of a protein in water) are measurements commonly employed when using protein concentrates to evaluate the extent of thermal processing.⁴⁸ Thermal processing is often used to decrease the content of antinutritional factors in OC. However, while under-processing treatments may not affect antinutritional factors concentration, overprocessing may affect protein stability, particularly available lysine. The KOH solubility ranging from 70 to 85 % is generally regarded as ideal for soybean meal reflecting a good quality protein.⁴⁹ The solubility of proteins could be altered during the heating, especially during the sterilization of OC leading to a denaturation of a small portion of proteins present in OC mixtures. The extraction of soluble compounds namely nonprotein N compounds after SSF resulted in a lower PDI of F_{EXT} mixtures which, compared with the respective F mixtures was an approximately 1.5-fold. The increases observed after SSF could be

attributed to the presence of nonprotein compounds that contribute to an increase of protein dispersibility.

Trypsin inhibitors (one of the most important antinutritional factor present in OC) are mainly found in SBC and they inhibit the action of proteolytic enzymes in the digestive tract reducing trypsin and chymotrypsin activity and consequently protein digestion.⁵⁰ The SSF led to an increase of trypsin inhibitors compared to non-fermented mixtures. However, according to Chen et al.⁵¹ the observed content of 0.3 % (w/w) for F_2 is within the recent indicators of analysed SBM in European countries. Trypsin inhibitors are water soluble compounds and this can explain the observed reduction of this parameter for F_{EXT} .⁵²

Although the presence of urea does not affect poultry productivity, urease enzyme can be inactivated by heat.⁵³ Therefore, urease activity can indirectly provide information on over or under-processing of the products and thus on the potential antinutritional factors present other than urea (such as trypsin inhibitors). Urease activities in excess of a 0.15 units suggest under processing (and thus potentially trypsin inhibitors remaining in the meal), while activities of less than 0.05 units indicated overprocessing.⁵⁴ An excess of heat, can increase the incidence of Maillard reactions, reducing the digestibility of amino acids like lysine and the nutritive value of OC.⁵³

Lysine is an essential amino acid and is the second limiting amino acid, after methionine, in poultry diets.⁵⁵ This amino acid is used as reference in feed formulations due to its importance in body protein deposition⁵⁶. Shi et al.⁵⁷ observed a decrease of lysine content after SSF of drumstick leaf flour with a microbial consortium composed by *A. niger*, *Candida utilis* and *Bacillus subtilis*. The inoculated microorganism may have used lysine for metabolic activity leading to lower concentrations in the fermented substrate compared to raw OC.

Overall, SSF was able to increase the nutritional value of OC mixtures in special of F mixtures resulting in fermented products with increased CP and minerals content, and decreased fiber fractions (except for lignin). This process was able to decrease antinutritional factors concentration successfully for phytic acid and glucosinolates (F_1 and F_{EXT1}). On the other hand, the aqueous extraction resulted in solid by-products (F_{EXT}) with increased concentration of fibre fractions and the same or a slightly higher content of minerals and CP than non-fermented mixtures. Additionally, our data shows that SSF can reduce the fiber content but may reduce the availability of CP through lower amino acid content, lower soluble protein, higher protein dispersibility and lower available lysine.

The inclusion of SFC in mixture 2 and consequently the lower proportion of SBC compared to mixture 1 led to lower digestibility values of NF mixture 2. Mixture 2 included 2/3 of highly fibrous protein concentrates (SFC and RSC) that impaired the overall digestibility of this mixture compared to mixture 1. From the three OC tested in this study, SFC has been reported as the one with a higher content of undigestible fiber, followed by RSC and SBC.⁵⁸

Through SSF it was possible to increase the DM digestibility of F mixtures 1 and 2 by 3.77 and 7.38 percentage points compared to the respective non-fermented mixtures. The increments of OM digestibility of fermented OC were lower compared to the DM digestibility but still statistically significant. The increase in OM was equal to 4.61 percentage points for F_1 compared to non-fermented mixture 1; and 8.50 percentage points for F_2 compared to non-fermented mixture 2. The OM fraction does not include minerals and inorganic compounds which in the *in vitro* systems are not retained while in the *in vivo* experiments these compounds could be absorbed by the animal.

In contrast to F mixtures, F_{EXT} showed lower digestibility values as a consequence of the aqueous extraction. This extraction removed fermentable carbohydrates and other soluble organic compounds (proteins, phenolic compounds, organic acids) from fermented OC obtained by the enzymatic hydrolysis of structural carbohydrates (cellulose and hemicellulose) and protein fractions of OC. As a consequence of the extraction process, F_{EXT} mixtures showed a concentrated fraction of highly undigestible fibre (lignin) compared to NF mixtures and this fraction is not degraded by endogenous digestive enzymes.^{59,60} DM digestibility of F_{EXT} in mixtures 1 and 2 was respectively 1.41 and 1.35-fold lower than the corresponding non-fermented mixtures. The reduction of OM digestibility for F_{EXT} was 1.12-fold for mixture 1 and 1.08-fold for mixture 2 compared to the corresponding NF mixtures.

The results of protein digestibility showed an increase in F mixtures compared to the corresponding non-fermented mixtures. Increases of 3.16 and 6.67 percentage points were observed for mixtures 1 and 2, respectively; although only F_2 showed a statistically significant increase compared to NF mixture. Also, the fermented ternary combination of OC (F_2) achieved protein digestibility values significantly higher than NF mixture 1 which had SBC concentration 1.5-fold higher than non-fermented mixture 2. The aqueous extraction resulted in lower digestibility coefficients of F_{EXT} mixtures because of the extraction of nonprotein N compounds obtained from the action of proteolytic enzymes during SSF. Reductions around 1.3-fold were observed for F_{EXT} mixtures. The inclusion of SFC in the ratio of 1:1:1 with RSC and SBC did not result in statistically significant differences in protein digestibility achieving similar values between the corresponding mixtures of NF, F and F_{EXT} (P-value treatment (NF, F, F_{EXT}) < 0.0001).

Increase in nutrient *in vitro* digestibility coefficients has been reported for SSF products in broilers in the literature. Although in *in vivo* conditions,⁶¹ reported an increase of CP digestibility from 57.92 % to 61.83 % after SSF of palm kernel cake with *Paenibacillus polymyxa* in broilers. Additionally, Hakim et al.⁶² also reported an increase in CP digestibility from 45.90 % to 59.90 % after SSF of palm kernel cake, using *Weissella confuse* in broilers, as well.

In vitro models are reliable and useful predictors of digestibility coefficients mimicking the animal's gastrointestinal tract, but they are not as complete as *in vivo* systems. Perhaps, the incubation time of exogenous enzymes with meals during the crop phase may not have been enough to efficiently degrade the cell wall components of OC.

5.5. Conclusions

The bioprocessing of OC mixtures through SSF was able to decrease fiber fractions (except for lignin), antinutritional factors and increase crude protein (1.24-fold) and mineral content (1.39-fold). However, this bioprocess compromised the protein quality and its bioavailability which resulted in lower amino acids concentration, lower soluble protein, lower available lysine, and higher protein dispersibility. This was attributed to a transformation of true protein into nonprotein compounds such as ammoniacal nitrogen. The aqueous extractions led to the obtainment of a concentrated protein fraction with highly undigestible fiber. The *in vitro* digestibility successfully reproduced the digestive system of poultry, and non-extracted solid-state fermented mixtures significantly increased the dry matter, organic matter, and crude protein digestibility coefficients compared with non-fermented. Both fermented mixtures achieved similar digestibility values despite the incorporation of a highly fibrous OC in the ternary mixture of OC. The enzymatic supplementation of raw OC in different concentration levels did not affect the digestibility parameters regarding crude protein, dry matter, or organic matter.

This work allowed the identification of SSF advantages on the reduction of antinutritional factors and increase of digestibility parameters and the identification of further improvement areas regarding the bioprocessing of proteinaceous sources namely in protein quality, when intended to be used in monogastric feed.

5.6. References

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Chapter 6

Evaluation of biotechnological processing through solid-state fermentation of oilseed cakes on extracts bioactive potential

This chapter was submitted to *Biotechnology Letters*:

Sousa, D.; Simões, L.; Oliveira, R.; Salgado, J. M.; Cambra-López, M.; Belo, I.; Dias, A. Evaluation of biotechnological processing through solid-state fermentation of oilseed cakes on extracts bioactive potential

Abstract

Oilseed cakes (OC) are natural sources of lignocellulosic biomass, produced every year in large amounts. In addition to their main applications as animal feed, plant, or soil fertilizer and for compost, they present enormous potential for being used in biotechnological processes for the obtainment and extraction of valuable bioactive compounds. Phenolic compounds are plants secondary metabolites, known for being responsible for bioactive properties including antioxidant, antimicrobial and antigenotoxic. This work evaluated the effect of solid-state fermentation in the bioactive properties namely antioxidant, antimicrobial and antigenotoxic of extracts obtained from the bioprocessing of OC and evaluated the effect of solvents on the recovery of compounds with higher bioactive potential. A general decrease of effective concentration (EC_{50}) values was observed for fermented extracts obtained using a mixture of water: methanol (1:1, v/v) as extraction solvent. A decrease of the minimum inhibitory concentration was observed for fermented water extracts compared to non-fermented. Additionally, it was observed the growth inhibition of *Listeria monocytogenes* when using aqueous methanolic fermented extracts. These also exhibited higher percentage of growth reduction against phytopathogenic fungi, and some extracts exhibited increased protection against genotoxic agents such as camptothecin and bisphenol A. This study demonstrated that bioprocessing of OC through SSF is an effective approach to obtain valuable compounds with bioactive properties for being used in the food, pharmaceutical or cosmetic industries.

Keywords: bioactive compounds, solid-state fermentation, extraction solvent, consortium, antioxidant , antimicrobial, antigenotoxic

6.1. Introduction

Lignocellulosic biomass (LB) is a reliable source of material for biotechnological processing for the obtainment of bioactive compounds. LB comprises a complex matrix, in which cellulose is wrapped by the dense structure formed by hemicellulose and lignin.¹ Cellulose is the most abundant natural biopolymer and is a water-insoluble homopolysaccharide composed of a linear chain of β – D – glucopyranose units linked by β – (1 $\rightarrow$ 4) - glycosidic bounds.^{2,3} Following, hemicellulose appears as the second largest natural biopolymer with an amorphous structure, more hydrophilic than cellulose and easier to hydrolyze,⁴ it is a heteropolymer composed by pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and uronic acids.^{3,5} On the other hand, lignin is the major non-carbohydrate compound of LB, it is a heterogeneous tree-dimensional network composed of a complex structure of aromatic compounds associated with both cellulose and hemicellulose. These fractions establish non-covalent bonds and covalent cross-linkages conferring the hard structure and stability, characteristic of lignocellulosic materials, and this complex intermeshed structure makes these materials recalcitrant to enzymatic degradation.⁶

A natural and abundant source of LB are the oilseed cakes (OC). The vegetable oils industry is responsible for the generation of large amounts of solid by-products called cakes, achieving a production value of 363 million tons during 2021.⁷ The OC are natural sources of LB and result from the extraction process of oil from oilseeds. OC are characterized by a high protein content, higher fiber fraction and they are natural sources of minerals, vitamins and phenolic compounds with bioactive potential properties.⁸ OC are normally used as animal feed with relative amounts of incorporation in animal feed rations, due to the presence of antinutritional compounds or high amounts of fiber that may hinder the digestive process.⁹ Additionally, OC can be used as plant fertilizer, plant/soil compost or for the obtainment of protein concentrates.^{10,11} Therefore, these OC combine the perfect environment for being used as raw material in biotechnology processes, however, due to their nature, a pre-treatment process is essential to overcome their complex structure and obtain maximum profit from their exploitation.

Biotechnological processing of LB has been reported as one of the most cost-effective ways to up-cycle by-products to obtain and recover valuable bioactive compounds (protein, peptides, enzymes, pigments, phenolic compounds) with economical and industrial interest while simultaneously obtain nutritionally up-graded products.^{12–14} Bioactive compounds are naturally occurring compounds, specially found in plants and food products,¹⁵ these compounds, present in residues matrices, can be potentially used for the prevention and/or treatment of diseases including diabetes, neurological diseases, and

cardiovascular diseases.^{16–18} Also, these compounds can be used for incorporation in food products contributing to the increase of the nutritional, sensorial and technological characteristics of a certain product.^{19,20}

The use of low-environmental impact bioprocesses such as solid-state fermentation (SSF) allow the depolymerization of the lignocellulosic matrix into more simple structures. SSF is a fermentation process that mimics the conditions present in natural *habitats* and in which microorganisms' grow in the absence or near absence of free water to which is applied a solid substrate used as support for the growth and development of microorganisms.²¹ The use of OC as substrate in SSF can be an effective strategy for the obtainment and recovery of valuable bioactive compounds.

Phenolic compounds are among the secondary metabolites produced by plants of greatest interest due to their bioactive properties such as antioxidant, antimicrobial, anti-inflammatory and anti-carcinogenic.^{22,23} The phenolic profile of LB sources such as the OC may vary depending on the oilseed variety, growing conditions and location and can include various chemical structures such as tocopherols, flavonoids, lignans, phenolic acids and tannins. Soybean cake (SBC), rapeseed cake (RSC) and sunflower cake (SFC) are the most produced OC at a global scale and chlorogenic acid, caffeic acid, synaptic acid, p-coumaric, trans-ferulic acid, hydroxybenzoic acid and isoflavones such as genistein and daidzein are the main compounds found in these OC.^{24–28}

The objective of this work was to evaluate the effect of SSF (single species or consortia of two species) on the bioactive properties of OC extracts using SFC and RSC as substrate. The single species was the fungus *Aspergillus niger* in SSF at the previous optimized substrate mixture of OC (SFC:RSC:SBC at 1:1:1, w/w). Additionally, the use of different extraction solvents was evaluated considering the bioactive properties of the final extracts.

6.2. Materials and methods

6.2.1. Oilseed cakes

Three oilseed cakes (OC) from the vegetable oils industry were used during this work: sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC). OC were obtained after cold-press extraction of oil and provided by industries operating in Portugal. SFC was provided by Sorgal, S. A. and RSC and SBC were provided by Iberol – *Sociedade Ibérica de Oleaginosas*, SARL. OC were dried for 24 hours at 65 °C and stored in hermetic bags in the dark at 20 °C. OC were previously characterized by Sousa et al.²⁹ Following previous studies three conditions were tested during this work and the effect of solid-state fermentation (SSF) on the bioactive properties of OC extracts was evaluated comparing the bioactivities

of non-fermented extracts with solid-state fermented extracts. The extracts used during this work and the process to obtain them are identified in **Figure 6.1.**

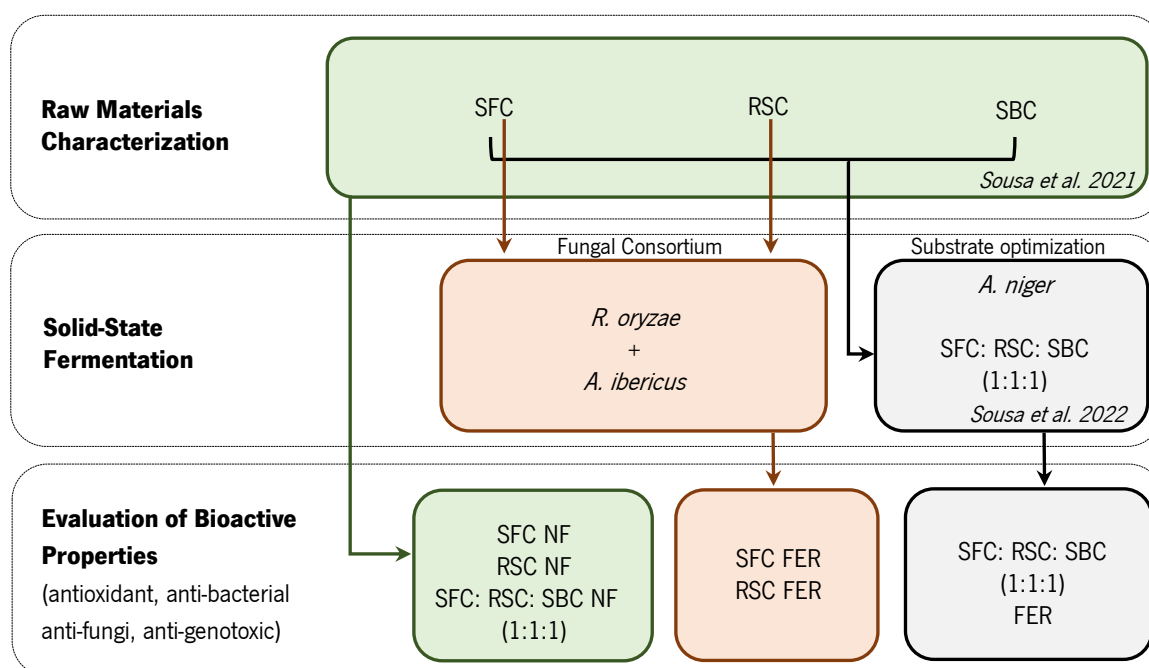


Figure 6.1. Schematic representation of extracts in study and the processes of how they were obtained. SFC, sunflower cake; RSC, rapeseed cake; SBC, soybean cake. NF, non-fermented, FER: fermented.

SFC and RSC were used to obtain extracts as well as its fermented biomass obtained by SSF with a microbial consortium composed by *Aspergillus ibericus* MUM 03.113 and *Rhizopus oryzae* MUM 10.260. Additionally, an optimum substrate that maximizes the production of lignocellulolytic enzymes while decreasing the fiber content of biomass, composed by the conjugation of OC (SFC:RSC:SBC in a ratio of 1:1:1) was also evaluated during this work.¹⁴ The non-fermented mixture of substrates was used for the obtainment of extracts as well as the fermented biomass obtained after SSF with *A. niger* CECT 2915 as described by Sousa et al.¹⁴

6.2.2. Tested extracts

A liquid/ solid extraction was performed to obtain liquid extracts. Extractions were performed in 1L Erlenmeyer flasks containing 50g of biomass. Water at 50 °C and a mixture of water: methanol (1:1, v/v) were used as extraction solvents in a ratio of 1:8 (solid: liquid). Mixtures were stirred for 1h in an orbital shaker at 20 °C and 150 rotations per minute. The mixture was filtered with a fine-mesh net and

the remaining liquid fraction was centrifuged at 2264 *g* for 10 min at 4 °C. Methanol present in extracts obtained using a mixture of water and methanol as solvent was recovered using a rotary evaporator at 40 °C. All extracts were freeze dried and stored in a desiccator until further use.

6.2.3. *In vitro* antioxidant activity

For the *in vitro* evaluation of the antioxidant potential, the lyophilized samples were resuspended in methanol and the antioxidant potential was assessed by five different assays using concentrations varying from 6.25 to 2000 µg mL⁻¹. The scavenging potential of free radicals was evaluated by the DPPH method as described by Dulf et al.³⁰ Extracts capacity to chelate the ferrous ion was evaluated by the iron chelating activity (ICA) and the extracts reduction potential was evaluated by the Ferric Reducing Antioxidant Power (FRAP) assay as described by Sousa et al.²⁹ with some modifications. The superoxide radical scavenging potential was evaluated by the PMS-NAD non-enzymatic assay as described by Gangwar et al.³¹ These assays were performed with some modifications as described by Sousa et al.²⁹ The scavenging potential of nitric oxide (NO) was evaluated according to Sarwar et al.³² with some modifications. Briefly, 50 µL of sample was mixture with 50 µL of 10 mM sodium nitroprusside (SNP) in phosphate buffer saline (PBS) and incubated for 140 minutes at room temperature. Then, 50 µL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride (NED), and 2% phosphoric acid, w/w) was added to samples and were kept in the dark for 10 minutes. Absorbance was measured for all assays using a spectrophotometer (SpectraMax Plus 384, Thermo-Fisher Scientific) and the results of extracts antioxidant potential were expressed as the concentration able to produce 50 % of the maximal response (EC₅₀).

6.2.4. Microdilution broth assay

Extracts were resuspended in ultra-pure sterile water, filtered through a sterilized syringe filters (0.20 µm) and stored in sterile amber glass vials. Extracts antibacterial activity was tested against three pathogenic bacteria: *Escherichia coli* CECT 426, *Listeria monocytogenes* CECT 4031T and *Staphylococcus aureus* ATCC 25923. Extracts antibacterial activity was also tested against *Bacillus subtilis* 48886. The minimal inhibitory concentration (MIC) was evaluated using a microdilution broth assay according to Wiegand et al.³³ The bacterial isolates to be tested were first grown in nutrient-rich agar Mueller-Hinton (Becton-Dickinson) to obtain single colonies. Three to five isolated colonies were then transferred to Mueller-Hinton broth and incubated overnight with agitation (200 rpm) at 37 °C and *B. subtilis* at 30 °C. After incubation, suspensions were centrifuged for 3 minutes at 4400 *g*. The

supernatant was discharged, and cells were washed and vortexed with a sterile saline solution NaCl 0.85 % (w/w). The suspension was centrifuged for 3 minutes at 4400 *g* and this procedure was repeated one more time. Cells were resuspended in Mueller-Hinton broth and the OD of the final suspension was adjusted to 0.08 - 0.13 at 625 nm. After OD adjustment, bacterial suspension was used within 30 min to prevent bacterial cell growth. In a microtiter plate 150 μ L of extract was added to each well and inoculated with 150 μ L of bacterial suspension. Microtiter plates were agitated to involve the extract and bacterial suspension and kept overnight at constant shaking (200 rpm) in a temperature-controlled incubator at 37 °C or 30 °C in the case of *B. subtilis*. Additionally, in the microtiter plates a growth control was included with 100 μ L of bacterial suspension and 50 μ L of Mueller-Hinton broth and a sterility control with 150 μ L of Mueller-Hinton broth. For each extract were tested concentrations ranging from 0.78 – 25 mg mL⁻¹. After 16-20 h of incubation and if no contamination was observed, 20 μ L of resazurin reagent (R7017, Sigma-Aldrich) was added to samples.³⁴ The plates were then placed in an incubator at 30 or 37 °C for 2 hours, all tests were performed in triplicate and the MIC was visually assessed by the color change of resazurin in each well (blue to pink in the presence of bacterial growth) ³⁵.

6.2.5. Antifungal activity assay

The effect of extracts on the mycelial growth of phytopathogenic fungi (*Botrytis cinerea*, *Colletotrichum nymphaeae* 15-019, *Diplodia corticola* CAA500, *Phytophthora cinnamomi* PH107) was evaluated in Petri dishes through its incorporation in the medium.³⁶ Extracts were resuspended in ultra-pure sterile water, filtered through a sterilized syringe filters (0.20 μ m) and added to PDA medium (Biolife Italiana, S.R.L.) at approximately 50 °C at a concentration of 1500 μ g mL⁻¹ and the final mixture was placed in 90 mm diameter sterilized Petri dishes. After agar solidification, inoculation with fungi was made with a 5 mm diameter mycelial disc of each fungus, removed from the margins of 12 days old cultures in PDA, in the center of the agar surface and incubated at 25 °C for 7 days. Water was used as negative control and added to PDA in the same amount as extracts. After 7 days of incubation the diameter of fungi colonies was measured, and the inhibitory activity of each treatment was expressed as the percentage growth inhibition compared to control using the following equation 6.1.:

$$growth\ inhibition\ (\%) = \frac{DC-DT}{DC} * 100 \quad (6.1.)$$

Where DC is the diameter of control, and DT is the diameter of fungal colony with treatment.³⁷

6.2.6. Antigenotoxic potential assay

The antigenotoxic potential of extracts was evaluated by a viability assay through the spot assay according to Fauzya et al.³⁸ with some modifications. The yeast culture of *Schizosaccharomyces pombe* wild type (wt) 972h- was prepared by inoculating a loop of yeast colony in YES liquid medium (5 g/L yeast extract, 30 g/L glucose, 225 µg/mL adenine, 225 µg/mL L-histidine, 225 µg/mL L-leucine, 225 µg/mL uracil, 225 µg/mL L-lysine hydrochloride). The culture was then incubated for 24 h in a shaker at 30 °C and 200 rpm and used as a subculture. The turbidity of the subculture was then measured with a final OD₆₀₀ of 0.4, to prepare the different conditions.

Aliquots were prepared for the different treatments: Positive control (yeast cells only in YES); Negative control (yeast cells in YES + 750 µg mL⁻¹ of solvent methanol); Genotoxic control (yeast cells in YES + 50 µg mL⁻¹ camptothecin or 0.75 mM bisphenol A); and the treatments (yeast cells in YES + genotoxic agent + extracts). Yeast cells were grown in the presence of 750 µg mL⁻¹ of each aqueous methanolic extract (fermented or non-fermented extract of sunflower, rapeseed, or soybean cake). Cells were incubated at 30 °C for 6 h at 200 rpm, the different cell suspensions were serially diluted up to 10⁻⁴, and 5 µl of each dilution was then placed on top of YES agar and further incubated at 30 °C for 72 h. A preliminary evaluation of the yeast growth in the presence of each extract was made, using Petri dishes.

6.2.7. Statistical analysis

Differences in extracts obtained from the same OC for evaluation of the effect of SSF were analyzed using one-way analysis of variance (ANOVA) at a significance level of 5%. All dependent variables were compared by a post hoc Tukey's Honestly Significant Difference (HSD) test, using Statgraphics Plus 5.1 (Manusgistics, Inc., Rockville, MD). The EC₅₀ value was obtained using GraphPad Prism software.

6.3. Results and Discussion

6.3.1. Antioxidant potential of non-fermented and fermented extracts

The effect of SSF and extraction solvent on the antioxidant potential of non-fermented and fermented extracts was evaluated. **Figures 6.2. and 6.3.** show the results of the concentrations able to produce 50 % of the maximal response for the different extracts. Considering the extracts obtained using water as extraction solvent (**Figure 6.2.**), the positive effect of SSF in the increase of antioxidant potential

was clearly observed using SFC as substrate. There was a decrease of 3.25-fold of the DPPH EC₅₀ comparing the SFC NF with SFC FER, this decrease is translated into a higher antioxidant potential from the fermented SFC. The other antioxidant activities showed no statistically significant differences ($P < 0.05$) between non-fermented and fermented extracts. Some variations of the antioxidant profile of RSC and the OC mixture were observed, SSF led to an increase of the EC₅₀ value of superoxide scavenging potential decreasing extracts capability to scavenge this radical when compared to RSC NF. On the other hand, with SSF the extracts reducing capacity increased resulting in a lower EC₅₀ comparing to RSC NF.

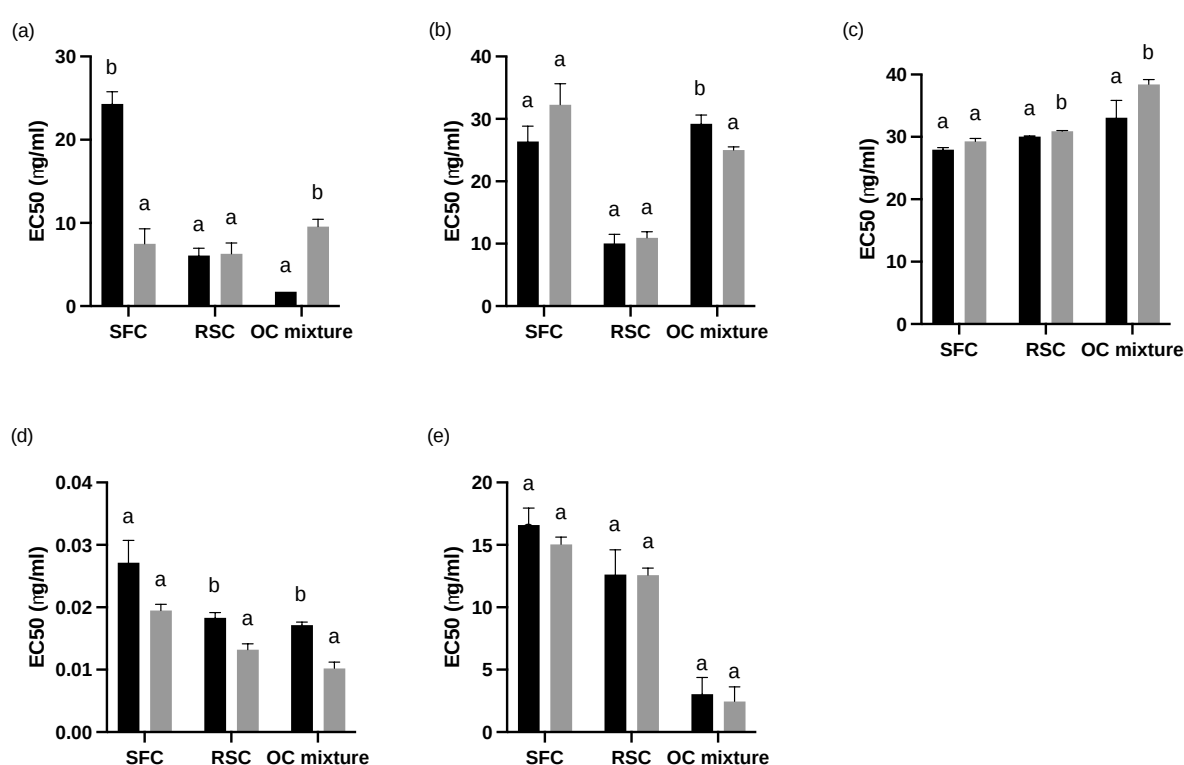


Figure 6.2. Antioxidant potential of non-fermented (black) and fermented aqueous extracts (grey). (a) DPPH radical scavenging activity; (b) iron chelation ability; (c) superoxide radical scavenging activity; (d) reducing ability (e) nitric oxide scavenging potential. SFC, sunflower cake; RSC, rapeseed cake; OC mixture, SFC: RSC: SBC (1:1:1). Results represent the average of three independent experiments and error bars represent standard deviation. Bars with equal letters for the same substrate are not statistically different (Tukey test; $P < 0.05$).

The scavenging potential of nitric oxide was the only antioxidant property that did not change with SSF with statistical significance, for the mixture of three OC as substrate. The NF mixture of OC exhibited a 5.46-fold higher scavenging potential of free radicals as well as a 1.16-fold higher scavenging of superoxide radical than fermented OC extracts. On the other hand, fermented OC extracts exhibited a 1.17-fold higher iron chelation potential than NF mixture of OC and a 1.7-fold higher reduction potential. The use of organic solvents or the mixture of different solvents for the extraction of phenolic compounds with bioactive properties has been reported by several authors.^{39–42} The use of a mixture of water: methanol (1:1, v/v) as extraction solvent resulted in an overall antioxidant profile with lower EC 50 values for fermented extracts compared to non-fermented (**Figure 6.3.**).

With SSF the scavenging of free radicals, reduction power and scavenging of nitric oxide radical of SFC FER were significantly ($P < 0.05$) increased, resulting in a lower EC 50 value for each antioxidant property, comparing to SFC NF extracts. The EC 50 of SFC FER was 3.18, 3.19 and 6.16-fold lower for the DPPH, FRAP and ON when compared to the respective SFC NF EC 50 values. Contrarily, SSF led to an increase of 1.45 and 1.12-fold of the EC 50 value of iron chelation potential and scavenging potential of the superoxide radical, respectively. The antioxidant profile of RSC shows increases of the scavenging potential of free radicals and superoxide radicals and a higher reduction potential of 2.29, 1.66 and 2.53-fold, respectively. On the contrary, the chelation potential and scavenging potential of the nitric oxide radical decreased 1.75 and 2.13-fold compared to SFC NF extracts. Non-fermented extracts obtained from the mixture of OC showed 1.66 and 2.13-fold higher scavenging potential of free radicals as well as superoxide anion, respectively compared to fermented extracts, however, the last presented 1.75, 1.91 and 5.52-fold higher chelation potential, higher reduction power and higher scavenging potential of nitric oxide, respectively. The SSF promoted variations on the antioxidant profile between non-fermented and fermented extracts that can be attributed to the deconstruction of the lignocellulosic matrix by fungi during SSF.⁴³

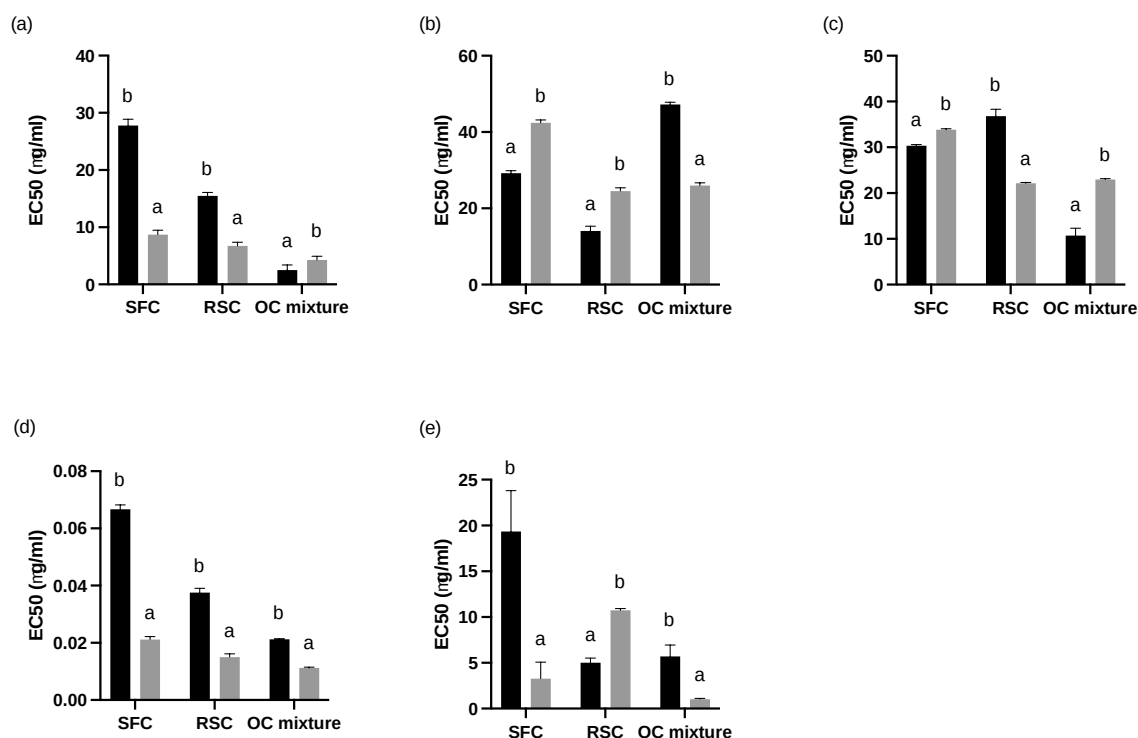


Figure 6.3. Antioxidant potential of non-fermented (black) and fermented aqueous methanolic extracts (grey). (a) DPPH radical scavenging activity; (b) iron chelation ability; (c) superoxide radical scavenging activity; (d) reducing ability (e) nitric oxide scavenging potential. SFC, sunflower cake; RSC, rapeseed cake; OC mixture, SFC: RSC: SBC (1:1:1). Results represent the average of three independent experiments and error bars represent standard deviation. Bars with equal letters for the same substrate are not statistically different (Tukey test; $P < 0.05$).

The vegetable matrix is rich in phenolic compounds, in special lignin. The action of exogenous enzymes may have contributed to the release of bound phenolics and lower molecular weight compounds with higher antioxidant potential.⁴⁴ Additionally, during fermentation the degree of glycosylation of phenolics changes affecting its bioactivity.⁴³

Overall, EC₅₀ values obtained using water as extraction solvent were lower than the EC₅₀ values obtained by conjugation of water: methanol (1:1, v/v). A lower value of EC₅₀ indicates a lower extract concentration to promote 50 % of the maximum response which can be translated into a higher potency of the extract compared to one with higher EC₅₀ values. However, it was observed statistically significant differences between NF and solid-state fermented extracts for all the antioxidant assays and substrates, using a mixture of solvents. This fact indicates that the conjugation of water with methanol allowed the recovery of lower polarity species that were not recovered during the water extraction. Magwaza et al.⁴⁵ reported that the use of conjugated solvents, more precisely de use of acidic aqueous

methanol (methanol:H₂O:HCl; 70:29.5:0.5, v/v/v), in comparison to the use of isolated solvents was a rapidly and efficient way of recovering phenolic acids and flavone glycosides with higher yields. Boeing et al.⁴⁶ also reported that the use of organic-solvent water mixtures was more effectively in the extraction of antioxidant compounds than their respective pure organic solvents, with acid or not. Phenolic compounds are normally responsible for the antioxidant potential of plant materials and they are mostly classified as hydrophilic antioxidants.⁴⁷ The use of methanol in conjugation with water as extraction solvent may have allowed the extraction of medium and low molecular weight polyphenols with antioxidant potential that were not recovered during the water extraction.⁴⁸

6.3.2. Antibacterial potential of non-fermented and fermented extracts

The bacterial inhibition potential of the extracts was evaluated against three pathogenic bacteria and *B. subtilis* through a microdilution broth assay with incorporation of resazurin, and the **Table 6.1.** depicts the results obtained.

Extracts obtained from aqueous extraction did not present any protection against the pathogenic bacteria in study (*E. coli*, *L. monocytogenes* and *S. aureus*), presenting inhibitory effect only to the microbial growth of *B. subtilis*. At an effective concentration of 12.5 mg mL⁻¹ both SFC NF and the NF mixture of substrates inhibited the microbial growth of *B. subtilis* while RSC NF extract showed higher efficiency of growth inhibition with a minimum inhibitory concentration of 6.25 mg mL⁻¹. Fermented aqueous extracts presented the same MIC of 3.13 mg mL⁻¹ for *B. subtilis*. These results indicate that SSF led to a decrease of the MIC, able to inhibit the growth of the bacteria *B. subtilis*.

Extracts obtained using water: methanol (1:1, v/v) as extraction solvent caused growth inhibition to *B. subtilis*, *E. coli* and *L. monocytogenes*. The MIC values observed for *B. subtilis* was 1.56 mg mL⁻¹ for SFC NF, and equal or higher MIC values of 6.25 mg mL⁻¹ was found to the other extracts. Also, it were not observed differences between non-fermented and fermented extracts of RSC and the mixture of the three OC. However, a MIC value 4-fold higher was obtained for SFC FER than for SFC NF, indicating that there was a loss of bacterial inhibitory potential due to fermentation. This fact may not be necessarily related with SSF as in aqueous extracts it was observed a reduction of MIC values for all extracts between NF and fermented ones. The aqueous methanolic extract obtained by the conjugation of the three substrates was the only one able to inhibit the microbial growth of *E. coli*, visual reduction of the MIC was observed between non-fermented (6.25 mg mL⁻¹) and fermented extracts (3.13 mg mL⁻¹). Non-fermented aqueous methanolic extracts were not able to have an effect in the growth inhibition of *L. monocytogenes*. Even tough, fermented aqueous methanolic extracts were able to inhibit the

growth of this bacterial species. Extracts from SFC FER and fermented OC mixture led to similar values of MIC while RSC FER result in extracts with the lowest value of inhibitory concentration.

Table 6.1. Antibacterial potential of non-fermented (NF) and fermented (Fer) extracts of oilseed cakes using microdilution broth assay. The represented values are the minimum extract concentration (mg mL⁻¹) able to inhibit bacterial growth. The absence of a concentration value means that the extract did not have any effect on bacterial growth within the used concentrations.

Solvent	Extract	Bacterial species			
		<i>B. subtilis</i>	<i>E. coli</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>
Water	SFC NF	12.5	-	-	-
	SCF FER	3.125	-	-	-
	RSC NF	6.25	-	-	-
	RSC FER	3.125	-	-	-
	OC mixture NF	12.5	-	-	-
	OC mixture FER	3.125	-	-	-
Water: Methanol (1:1, v/v)	SFC NF	1.5625	-	-	-
	SCF FER	6.25	-	12.5	-
	RSC NF	6.25	-	-	-
	RSC FER	6.25	-	1.5625	-
	OC mixture NF	6.25	6.25	-	-
	OC mixture FER	6.25	3.125	12.5	-

Overall, the aqueous methanolic extracts exhibited higher growth inhibition against pathogenic bacteria and, in the majority of the cases, SSF was able to increase the antibacterial effect of extracts. The use of OC as substrate in biotechnology processes for the production of antibiotics has been previously reported. Antibiotics can be produced by different microorganisms which selectively kill or inhibit growth in low concentrations.⁴⁹ Sircar et al.⁵⁰ reported the production of clavulanic acid and cephamycin using sunflower, soybean and sesame oilseed cakes as substrate for SSF. Sarada & Sridhar,⁵¹ reported the use of SFC for the production of cephamycin C. The fungi belonging to the genus *Aspergillus* and *Rhizopus*, used in SSF for the obtainment of the extracts in this study have been previously reported due to their antimicrobial potential.⁵²⁻⁵⁴ During the fermentative process fungi are able to produce compounds with antimicrobial activity which results in the higher antimicrobial potential observed for

the extracts in study, compared to non-fermented ones.⁵⁵⁻⁵⁸ Feitosa et al.⁵⁹ reported antimicrobial activity of fermented extracts against *B. subtilis* after SSF of moringa leaves flour with the fungus *A. niger* while the unfermented extracts did not exhibit the same bioactivity. The bioactive compounds produced by the studied strains showed anti-bacterial effect against both Gram-positive and Gram-negative bacteria, despite the complex structure and arrangement of the Gram-negative cell wall compared to Gram-positive bacteria.⁶⁰ These results indicate that the studied extracts show a high antimicrobial spectrum which increases their biotechnological applications.

6.3.3. Antifungal potential of non-fermented and fermented extracts

Non-fermented and fermented extracts revealed antifungal properties against the phytopathogenic fungus *Botrytis cinerea*, *Colletotrichum nymphaea*, *Diplodia corticola* and *Phytophthora cinnamomi*. The values of the percentage of growth inhibition induced by each extract are represented in **Table 6.2**. RSC NF was the only aqueous extract showing antifungal properties against the fungus *B. cinerea* leading to a halo reduction of 11 %. On the other hand, the aqueous methanolic extract obtained from RSC NF did not show antifungal properties, while the fermented (RSC FER), was able to inhibit the fungus growth by 61 %, being the maximum inhibition growth observed for this fungus. The higher percentage values of growth inhibition for *C. nymphae* and *D. corticola* were observed in both RSC fermented extracts namely in the aqueous and aqueous methanolic. Also, RSC FER aqueous methanolic extract led to a higher percentage of growth inhibition of *D. corticola* and *P. cinnamomi*.

According to the fermentation process, the fermented extracts exhibited higher percentage of growth inhibition compared to the respective non-fermented extracts. SSF had a positive effect in the increase of this extracts antifungal properties, as it is possible to observe by the percentage of growth inhibition of the aqueous extract of RSC FER corresponding to an inhibition percentage 2.4-fold higher compared to the non-fermented extract for *C. nymphae*. The aqueous extract obtained from the fermented mixture of OC presented a percentage of growth inhibition 1.4-fold higher than the corresponding non-fermented extract for *C. nymphae*. Compared to aqueous extracts, it was observed a higher fungal inhibition potential from aqueous methanolic extracts. RSC FER was able to induce a fungal inhibition growth 2.3 and 4.9-fold higher than RSC NF for *C. nymphae* and *D. corticola*, respectively. The increase of antifungal properties as consequence of SSF was also observed for the phytopathogenic fungus *P. cinnamomi* in RSC aqueous extracts where aqueous RSC FER extracts induced a inhibition growth 3-fold higher than RSC NF, Therefore, the effect of SSF in the increase of extracts potential against phytopathogenic fungi is clearer in RSC extracts and in extracts obtained from the mixture of OC. Non

fermented extract of SFC was the only able to confer protection against two of the fungi in study (*C. nymphaea* and *D. corticola*).

Table 6.2. Antifungal activity of non-fermented (NF) and fermented (Fer) extracts of oilseed cakes. Values represent the reduction percentage between the halo observed in petri plates with each fungus in the presence of OC extracts against the control (fungus in the presence of solvent). The absence of values indicate that the extract did not present antifungal effect.

Solvent	Extract	Fungal species			
		<i>Botrytis cinerea</i>	<i>Colletotrichum nymphaea</i>	<i>Diplodia corticola</i>	<i>Phytophthora cinnamomi</i>
Water	SFC NF	-	17	10	-
	SCF FER	-	-	-	-
	RSC NF	11	13	10	7
	RSC FER	-	31	13	21
	OC mixture NF	-	16	14	13
	OC mixture FER	-	22	13	-
Water: methanol (1:1)	SFC NF	-	18	16	-
	SCF FER	-	-	-	-
	RSC NF	-	21	11	-
	RSC FER	61	48	54	43
	OC mixture NF	20	11	-	-
	OC mixture FER	-	-	-	20

Phytopathogenic fungus are plant invaders that may led to plant diseases affecting crops yield and quality and are an increasingly concern for the development of sustainable agriculture.⁶¹ The obtainment of natural extracts with potential anti-fungal activity may decrease the application of fungicides with chemical origin which can lead to environmental and health problems.⁶² SSF has been used for the obtainment of fermented extracts with anti-fungal properties using a variety of substrates, Christ-Ribeiro et al.⁶³ reported a 39.8 % of inhibition growth of the fungi *Penicillium verrucosum*, after SSF of rice bran with the fungus *R. oryzae*. Denardi-Souza et al.⁶⁴ also reported anti-fungal properties of solid-state fermented extracts of rice bran with the fungus *R. oryzae*, against the genera *Aspergillus*, *Penicillium*, and *Fusarium*. The bioprocessing of oilseed cakes led to an increase of the anti-fungal activity of extracts increasing their potential applications namely in the agriculture area to act as plant protectors

against phytopathogenic fungi. These results highlight the importance of bioprocesses for obtaining value-added products with industrial applications and the potential of OC as raw materials.

6.3.4. Antigenotoxic potential of non-fermented and fermented extracts

Based on the previous results of extracts antioxidant and antimicrobial potential, the aqueous methanolic extracts were used for the evaluation of their geno-protective potential. The effect of SSF in the geno-protective effect of extracts was evaluated by comparison of the genotoxic protection of non-fermented extracts against the genotoxic protection of fermented extracts, for the same substrate used for SSF. The results of the spot assay for the genotoxic agent camptothecin are shown in **Figure 6.4.**, and for the genotoxic agent bisphenol A (BPA) the results are shown in **Figure 6.5.** Methanol and all the tested extracts had no effect in the growth of *S. Pombe* (not shown).

Through **Figure 6.4.**, it is possible to observe that non-fermented SFC extract showed an aggravation of the genotoxic effect of camptothecin, where in the presence of this extract and camptothecin, there was an intensification of the damage caused by this genotoxic agent, being able to observe a less growth of the yeast when compared with the growth in the condition only with the genotoxic agent. For the other tested extracts, the protection effect of the yeast cells was slightly noticed when in the presence of non-fermented RSC, fermented RSC or fermented OC Mixture since some damage mitigation effect was observed in these treatments.

Considering the use of BPA as genotoxic agent (**Figure 6.5.**), only in the presence of non-fermented RSC it was observed damage mitigation effect. In this case, it is possible to observe a higher yeast growth when in the presence of non-fermented RSC extract and the genotoxic agent BPA, when compared with the growth in the condition only with the genotoxic agent.

Exposure to genotoxic agents may result in DNA or chromosomal damage therefore, the continuous search for alternative compounds with biological activity capable of mitigating the harmful effects of external agents on our health must be continuous. Since camptothecin and BPA have adverse effects on the human body, it is important to identify natural compounds able to mitigate the harmful effects of these agents. The adverse effect caused by camptothecin affects topoisomerase I, allowing the cleavage of DNA but inhibits the subsequent ligation resulting in DNA double-strand breaks.⁶⁵ This agent is a plant alkaloid, widely used as anti-tumor drug in chemotherapy.^{66,67}

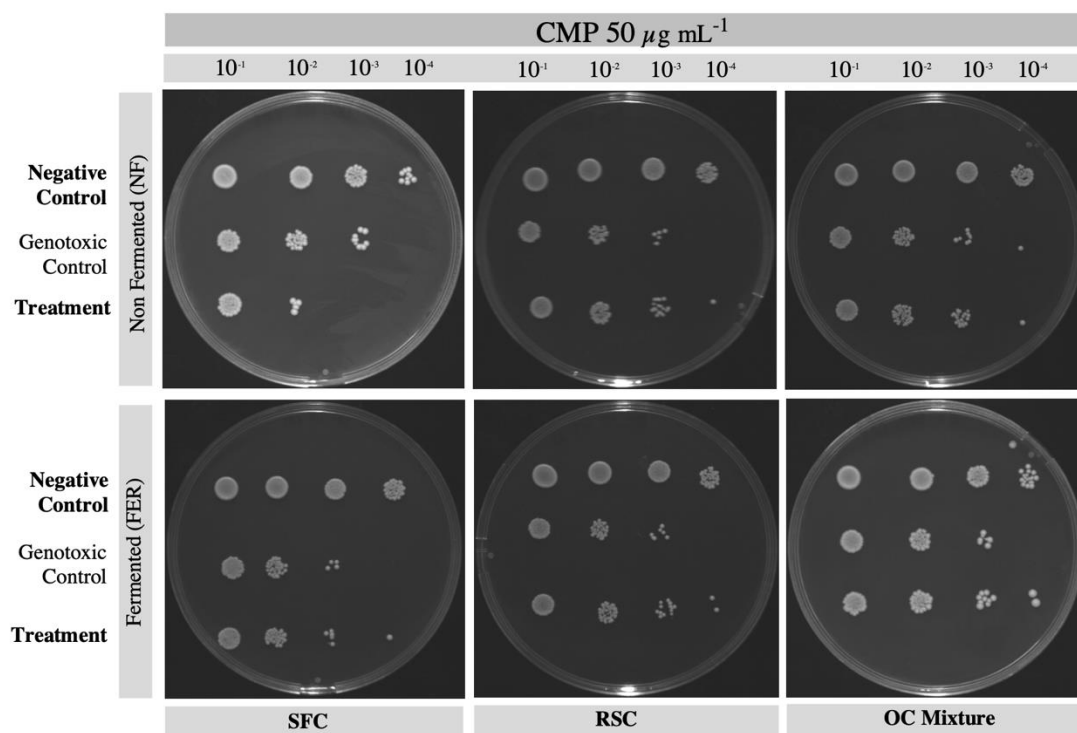


Figure 6.4. Spot assay used in viability assay performed with *Schizosaccharomyces pombe* in the presence of camptothecin and non-fermented or fermented oilseed cake extracts. Cells were grown at 30 °C for 6 h at 200 rpm. Positive control contained yeast cells grown in YES medium; Genotoxic control contained yeast cells in YES medium and 50 $\mu\text{g mL}^{-1}$ of camptothecin; the Treatment contained yeast cells in YES medium, the genotoxic agent and 750 $\mu\text{g mL}^{-1}$ of extract. Each figure represents the test of a different extract, and each column represents serial dilutions from 10⁻¹ until 10⁻⁴. The photographs shown are representative of three independent experiments.

Regarding BPA, this organic compound is used for the production of polycarbonate plastic and epoxy resins which are part of the packaging materials for food and beverages and can migrate to foods and the environment.^{68,69} It is believed that BPA has endocrine disruptive effects on humans by interacting with biological receptors such as estrogen, androgen and thyroid hormone receptors, which results in health hazards effects for the reproductive and nervous systems, metabolic function, immune function and interferes with growth and development of offspring.^{70,71} Besides, several authors have reported the genotoxicity of BPA namely in the impair of the double-strand break repair mechanism and the toxicity of this agent on the yeast *S. pombe*.^{69,72-74} Initiatives to reduce the exposure to these agents are essential to mitigate their harmful effects to the human body however, the use of biotechnological processing for the obtainment of extracts with geno-protective effect is an additional protection. In this innovative

study, some of the extracts exhibited geno-protection, being able to mitigate the effects of camptothecin and BPA widening the application of these extracts to the pharmaceutical industry.

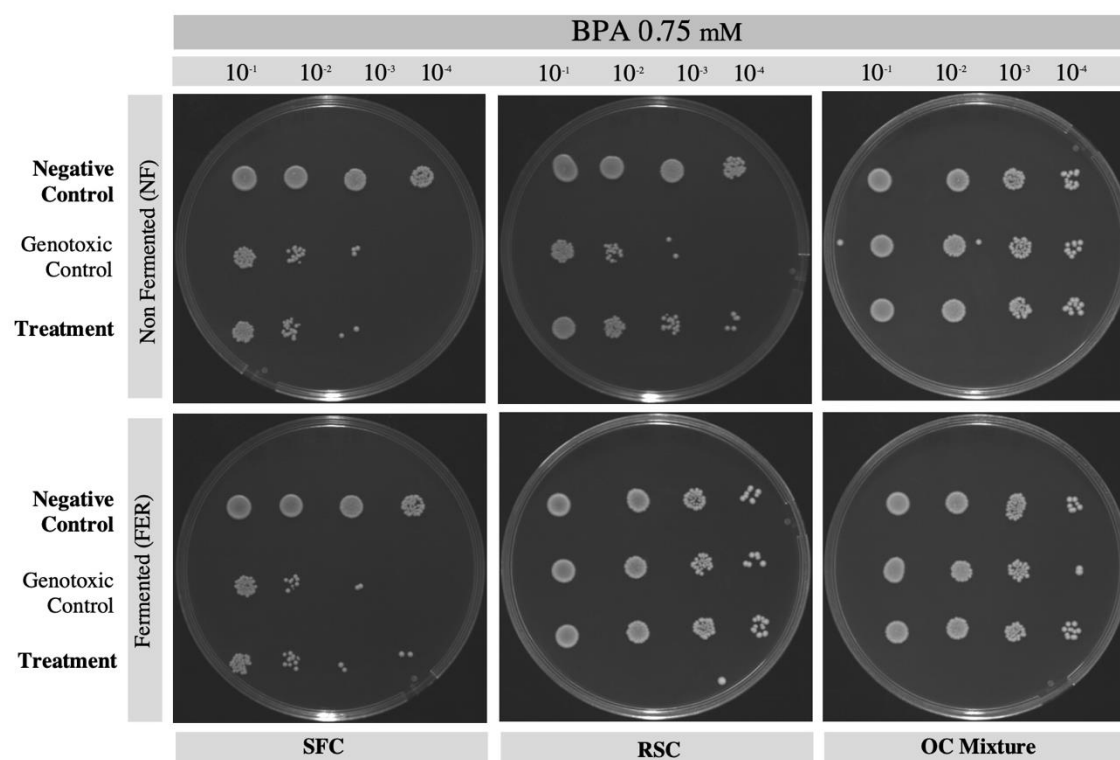


Figure 6.5. Spot assay used in viability assay performed with *Schizosaccharomyces pombe* in the presence of bisphenol A and non-fermented or fermented oilseed cake extracts. Cells were grown at 30 °C for 6h at 200 rpm. Positive control contained yeast cells grown in YES medium; Genotoxic control contained yeast cells in YES medium and 0.75 mM of bisphenol A; the Treatment contained yeast cells in YES medium, the genotoxic agent and 750 $\mu\text{g mL}^{-1}$ of extract. Each figure represents the test of a different extract, and each column represents serial dilutions from 10^{-1} until 10^{-4} . The photographs shown are representative of three independent experiments.

6.4. Conclusions

Oilseed cakes bioprocessing by SSF allowed the obtention of extracts with enhanced bioactive potential. The microbiological disruption of OC lignocellulosic matrix contributed to higher bioactive properties. The extraction method of solid-state fermented OC was optimized concluding that the aqueous methanolic solvent mixture allowed the recovery of compounds with lower polarity that contributed to the increase of the overall protection against certain agents including microbial, genotoxic and oxidants. This is a step further for the establishment of bioprocesses, namely SSF, as a reliable alternative for the obtainment of compounds with increased bioactive properties contributing to the up-grading of by-products and wastes within the concept of circular economy. Deeper studies are needed to test the

efficiency of extracts conferring protection of cell lines using *in vitro* trials against harmful agents. Nevertheless, this study demonstrated the potential of fermented extracts for industrial applications in the areas of food, cosmetics, agriculture and pharmaceutical.

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Chapter 7

Conclusions and Future Perspectives

Conclusions

OC are natural sources of lignocellulosic biomass with high nutritional and bioactive properties. Also, these products have great potential to be further valorised by biotechnological processing. This doctoral project aimed to design a strategy for the valorisation of OC and production of bioactive compounds within the concept of biorefining and according to the concept of circular economy.

In this work, it was proven that SFC, RSC and SBC have suitable chemical composition for being used as substrate in SSF without the need of pre-treatments or external supplementation. The degradation of the lignocellulosic fractions of OC by fungal extracellular enzymes was demonstrated and led to the release of phenolic compounds with antioxidant capacity. Among the three fungal species tested, *A. niger* led to the highest production of enzymes as well as antioxidants, indicating its great potential to the valorisation of these type of by-products by SSF.

Microbial consortiums of the fungal species have proven to be a suitable approach to apply in SSF of OC leading to extracts with higher enzymatic activity, compared to the reported by isolated cultures. On the other hand, optimisation design was successfully applied to find the OC mixture that maximized the target variables, namely enzymes (ex: carbohydrases) production and nutritional properties. This result highlights the importance of substrates mixture to respond to microorganisms' requirements regarding their nutrition, since single substrates may not have the optimal composition to the metabolic needs of microorganisms leaving enzymatic production below its maximum potential.

With this work it was possible to go a step further considering the nutritional properties of fermented by-products. Despite the observed increases in crude protein which normally is related as a positive effect of SSF, after nutritional analysis it was observed the production of non-protein compounds such as ammoniacal nitrogen and decrease of true protein. This is a limiting factor when considering animal nutritional as, among other factors, the protein quality was affected by the biotechnological processing. This aspect deserves further considerations and analysis as the SSF was able to increase digestibility coefficients of OC when compared to non-fermented OC. Also, SSF was able to increase the digestibility coefficients of the ternary mixture of OC to levels compared to the mixture of RSC: SBC (1:1), despite the presence of 2/3 of highly fibrous OC in the ternary mixture, compared to the last.

Finally, fermentation of OC led to extracts with higher bioactive potential compared to non-fermented extracts. The effect of SSF was again in evidence showing promising results that should be explored in further works. Globally, with this work it was demonstrated the potential of a simple and eco-friendly strategy, the solid-state fermentation, for the valorisation of OC, by OC biomodification, either by the

simultaneously production of value-added compounds with applications in several industries, including in animal feed.

Future Perspectives

The work carried out and discussed in the present dissertation focused on the importance of increasing the potential of lignocellulosic industrial by-products, from the vegetable oils industry and their possible applications. Although, considering the main results and conclusions, it would be interesting to further exploit the following topics:

- Apply the optimized fermentation medium in an additional scale-up study with other bioreactors (e.g., rotary drum) and increase the amount of solid used as substrate;
- Further explore the potential of fungal consortia in OC mixtures, e.g., design optimization procedures including both different fungal species and OC;
- Evaluate the potential of incorporation of fermented OC in animal feed formulations, e.g., to perform *in vivo* studies and compare with the *in vitro* results, namely in monogastric animals where the lignocellulosic fibers are difficult to digest;
- Characterize the phenolic profile of non-fermented and fermented OC extracts to evaluate the effect of SSF on the release of phenolic structures from OC matrix;
- Correlate the phenolic compounds present in non-fermented and fermented OC extracts with their bioactivities;
- Evaluate the bioactive potential of extracts using animal cell lines in *in vitro* models.