Universidade do Minho
Escola de Engenharia

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Despacho RT - 31 /2019 - Anexo 3

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Foi um longa jornada. 6 anos. 6 anos da minha vida passados nesta academia. Cresci como pessoa, conheci-me a mim mesmo, e fiz amigos para a vida, pessoas que sei que posso sempre contar, desenvolvi capacidades e competências que eu mesmo duvidava ter. Solucionei problemas, cultivei a minha independência, tive medos, receios e ansiedades, muitas das vezes injustificáveis e excessivas, mas isso também faz parte. Acima de tudo, fui feliz e saio daqui um ser humano melhor do que aquele que era há 6 anos, quando entrei num curso que nem tinha bem a certeza.

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"Would I rather be feared or loved? Easy. Both. I want people to be afraid of how much they love me." – Michael Scott

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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.

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Resumo

Fermentação em estado sólido para adicionar valor aos bagaços de oleaginosas

Os resíduos agroindústrias têm sido cada vez mais considerados um grande problema para a sociedade moderna, no entanto por causa da constante evolução e modernização científica e tecnológica, estes resíduos são agora vistos como um importante recurso que pode ser valorizado, perfeitamente de acordo com uma economia circular. Os bagaços de oleaginosas representam uma parte destes recursos, as suas características permitem que sejam reutilizados em muitos processos alternativos de recuperação e valorização, apesar de serem tratados como subprodutos pela indústria que os produz

Fermentação em estado sólido é um exemplo destas alternativas de valorização, que ao longo dos últimos anos tem atraído cada vez mais interesse. Neste processo biotecnológico, os bagaços são utlizados como substrato e suporte sólido para o crescimento dos microorganismos e concomitante produção de componentes com valor acrescentado.

Neste trabalho testou-se a suscetibilidade do bagaço de azeitona, juntamente com bagaços de girassol e de colza em serem aplicados como substrato para a produção de enzimas, através da fermentação em estado sólido usando a levedura *Candida cylindracea* e o fungo filamentoso *Trichoderma harzianum,* variando fatores como a composição do substrato (misturando bagaços), % de humidade e tempo de fermentação.

Valores máximos de atividade enzimática de 12 ± 2 U/g, para a lípase e 35.7 ± 2.6 U/g para a protease foram os alcançados pela *C. cylindracea.* Por sua vez, os valores obtidos utilizando *T.harziamum* foram maiores do que com a levedura, e várias outras enzimas foram também detetadas. Através de um desenho experimental de Box-Benchen, valores ótimos para a lípase, protease, celulase, xilanases, β-glucosidase e fitase foram determinados como 14.2 U/g, 108.3 U/g 10.9 U/g, 40.7 U/g 7.2 U/g e 73.7 U/g, respetivamente

A produção de variadas enzimas utilizando estas misturas de bagaços, possibilita a sua valorização nutricional e ajuda a validar a ideia de economia circular, de reutilização de subprodutos agroindustriais para o processo de fermentação em estado sólido, facilitando a possibilidade de, nas condições corretas, o bagaço de azeitona ser utilizado nas dietas animais.

Palavras-chave: Bagaços de oleaginosas, *Candida cylindracea*, fermentação em estado sólido, *Trichoderma harzianum.*

Abstract

Solid state fermentation to add value to vegetable oil cakes

Agro-industrial residues have been considered a major problem for modern society, but due to the constant scientific and technological evolution and modernization, these residues are now seen as important resources that can be valorized in full agreement with the concept of circular economy. Vegetable oil cakes represents a part of these resources, and their characteristics allow them, to be often reused in many alternative recovery and valorization processes, thus treated as by-products by the industry that produces them.

Solid state fermentation is an alternative valorization approach that has attracted more and more interest over the last few years. In this biotechnological process, oil cakes are used as a substrate and solid support to microbial growth and concomitant production of added value compounds.

In this work, was tested the suitability of olive pomace, together with sunflower and rapeseed cake to be applied as substrate for the production of enzymes, through solid state fermentation using the yeast *Candida cylindracea* and the filamentous fungus *Trichoderma harzianum*, varying factors such as substrate composition (by mixing oil cakes), % of moisture and fermentation time.

Maximum enzymatic activity values of 12 ± 2 U/g for lipase and 35.7 ± 2.6 U/g for protease were achieved by *C. cylindracea*. While with *T.harziamum* values of lipase and protease activities were higher than for the yeast, and several other enzymes were also detected. Through a Box-Benchen experimental design, optimal values of lipase, protease, cellulase, xylanase, β-glucosidase and phytase were found as 14.2 U/g, 108.3 U/g 10.9 U/g, 40.7 U/g 7.2 U/g and 73.7 U/g, respectively.

The production of various enzymes using these vegetable oil cakes mixtures enhances them nutritionally and helps to validate the idea of circular economy, of reusing agro-industrial by-products for the solid state fermentation process, facilitating the possibility of, under the correct conditions, use the olive pomace in animal diets.

Key-words: *Candida cylindracea*, solid state fermentation, *Trichoderma harzianum*, vegetable oilcakes

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List of abbreviations and acronyms

BBD - Box-Benchen experimental design

COP - Crude olive pomace

GHG - Greenhouse gases

GRAS - Generally recognized as safe

LBB - Laboratory of bioprocesses and biosystems

MEA - Malt extract agar

OC - Oil cakes

OD - Optical density

OP - Olive pomace

PDA - Potato dextrose agar

pNPG - 4-nitrophenyl β-D-glucopyranoside

RS - Reducing sugars

RSC - Rapeseed cake

SP - Soluble protein

SD - Standard deviation

SFC - Sunflower cake

SmF - Submerged fermentation

SSF - Solid state fermentation

YPDA - Extract peptone dextrose agar medium plate

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1. Introduction

1.1 Agro-industrial wastes

We currently live in an era of constant change and, while technological, scientific and healthcare developments help to solve several problems in modern society, many other situations that are difficult to resolve arise. Three of the most debated issues nowadays by world leaders and activist are human overpopulation, world hunger and pollution. All these issues are related, and as time goes on, becoming more and more serious threats to modern society.

It is a fact that the population have been increasing exponentially, so their constant demand for food supplies is leading to a boost in the production in the agroindustrial sector and others food related sectors, which overall, pointed to an inflation in the quantity of waste produced. In 2020 2.01 billion tonnes of municipal solid waste were produced with at least 33% of that (extremely conservatively), not managed in an environmentally safe manner. Nonetheless, even higher waste production is expected in the next decades (3.40 billion tones by 2050) owing to the constant growth of the world population and the subsequent increase in food production [1]–[3].

Agroindustrial residues are an important part of these solid wastes and do not escape this tendency. Despite the growing interesting and proposals of the applications of these by-products most of them are disposed in an untreated and underutilized way. Such as incineration, dumping or uncontrolled landfilled. This procedures cause environmental pollution and very serious and harmful effects on human and animal health [4].

Presently, the industries that produces the most waste excesses is the drink industry (26%), followed by the dairy and ice cream industry (21%) the vegetable/fruit production and processing industry (14.8%), followed by the cereal production industries (12.9%), meat production (8%), vegetable and animal oil processing (3.9%) and others with consecutively lower numbers [5].

The wastes particularly used in this work were: olive pomace (OP), sunflower cake (SFC) and rapeseed cake (RSC). These solid by-products are generated from the extraction of vegetable oils, respectively from the extraction of olive oil, sunflower oil and rapeseed oil. These vegetable oils have a very important role on human diet since they are ones of the most important sources of calories and energy in the human diet [6]. A general and average characterization of these agroindustrial wastes available in recent literature is shown in **table 1.**

Table 1. Composition of the vegetable oil cakes reported in the literature.

Substrate composition (g/kg)	Oil cakes		
	OP (1)	RSC (2,3)	SFC (2,4,5)
Crude protein	58 ± 4	338 – 403	236 – 525
Nitrogen	9 ± 0	75	47
Carbon	460 ± 10	502	498
Hemicellulose	289 ± 1	20	44 – 154
Cellulose	129 ± 2	59 – 82	123 – 251
Lignin	550 ± 20	64 – 90	77 – 133
Lipids	40 ± 2	118 – 167	28 – 303
Free sugars	33 ± 2	-	-
Phenols	7 ± 0	-	-
Ash	34 ± 2	43 – 70	52 – 75

(1) Leite *et al.*, 2016; (2) Sousa *et al.*, 2021 (3) de Castro *et al.*, 2016; (4) Halmemies-Beauchet-Filleau *et al.*,2018 (5) Tay *et al.,* 2009 [14], [34], [113]–[115]

Currently there are already some direct applications for these by-products, mainly for SFC and RSC, due to their richer composition (**Table 1**). They can be used as natural source of antimicrobials compounds [7], [8], for microbial pigments production [9], or as possible substrate for making of mushrooms as foodstuffs and other bio-based products like bio-energy and biofertilizers or even directly as animal food without no intermediate steps [4].

However, there are also some problems associated with some of these solutions, like the emissions of greenhouse gases (GHG) in biofuels application, although in smaller amounts than using fossil fuels for energy production. Also, the feed application is limited due to the poor nutritional quality that many of these residues offer to animal diets, since some do not meet the required standards for proper animal feed, due to low protein content or low digestibility, explained by the presence of polysaccharides, indigestible fiber and lignin in their composition [6], [10]–[12].

Regarding to olive pomace, a very problematic solid agroindustrial waste and the main focus of valorization in this work, many alternatives have been studied recently in order to counteract the growing problematic of waste management related with olive oil industry.

From the traditional 3-phases process of production of olive oil, it results the olive pomace, wastewaters, the olive pits and the olive leaves. These residues have a pollutant load 200 to 400 times higher than household waste [13].

More recently, the olive mills have changed their traditional extraction method from the three-phase system to the two-phase system [14], [15]. This change in the extraction process reduced water consumption, wastewater generation and improved the olive oil quality [16].

However, it generates a wet olive pomace (OP) or crude olive pomace (COP) whose handling is more difficult and cause more disposal problems than the pomace obtained by the 3-phases system [17].

Therefore, on average, the annual olive campaign in Portugal is equivalent to the garbage produced by 2.5 million people and if currently the olive oil industry already presents some solutions for the reutilization of almost all the wastes produced. Whether for the production of biomass or animal feed, many of them are ineffective in quantity or quality, and still very expensive.

Currently, most of the wastes produced in this sector are sent to the refinery industry, which in turn removes the remaining oil from the wet OP for the production of bagasse oil and proceeds to burn the rest, once again, causing a serious environmental problem [13].

Taking this into account, every year are observed problems related to excessive waste, lack of capacity to store and treat it, and environmental associations complaining about the harm and pollution that these by-products represent [15], [18], [19].

In order to respond to this social economic and environmental problematic and additionally valorize these agro-industrial wastes, providing the reduction of their negative impact emission, discharge, or disposal, as well as generating additional income, emerges the idea of reutilization and proper treatment of these by-products by fermentation with suitable microorganisms [20].

Fermentation processes with microorganisms intends to be an aid in the treatment of these residue, using them as a solid substrate alongside with other residues.

Since olive pomace alone does not show the necessary nutritional capacity to serve as good substrate or even to be used in animal feed due to high oil content, phenolic compounds and fiber contents in addition to its low protein content. Mixed with others and more suitable substrates, this fermentation can achieve the purpose of producing value-added products, mainly enzymes and serving

as a direct treatment of these by-product making them nutritionally richer and capable of being used in animal feed [21]–[23].

This possible reutilization and treatment can be extended to almost all of the residues with origin in the agro-industrial sector, and like this, help to reduce production costs often associated with fermentation processes, and significatively helping to reduce the levels of pollution generated by the industries that produces them [4], [24], [25]. Furthermore, it is a circular economic process, economically viable and helpful to society [26].

Recent studies reported the use of a wide range of organic residues, available as cheap substrates, in fermentation processes, these wastes include: rice bran, wheat bran, grape pomace, brewer's spent grain, oilseed cakes, coffee husk, sunflower cakes, wood chips, and olive pomace [11], [22], [27]–[30].

Although these substrates, have little value for the agro-industrial sector that produces them, considering them by-products ready to be dumped or burned with possible implications for the nature and their habitants, they have proved to be rich in bioactive and nutritional compounds, such as polyphenols, carotenoids and dietary fibers, cellulose, hemicellulose, lignin, starch, pectin and many other fibers [24], [25], [31], and can be reused almost directly in fermentation processes that can differ between SmF (Submerged fermentation) and SSF (solid state fermentation), serving as a substrate for various researches and development of new biocompounds and value-added products, such as enzymes, since most of these wastes and by-products favor the growth of microorganisms due to their lignocellulosic nature. [4], [31]

1.2 Solid state fermentation

By definition, SSF is recognized as any biotechnological processes in which organisms grow on non-soluble material or solid substrates (solid matrix) in the absence, or near absence of free water [20], [32], [33].

Agro-industrial wastes have been used in the recent years in SSF not only as an inert support but also as a source of nutrients for microbial growth. These wastes have the characteristics that makes them a good substrate for SSF with the additional cheapness and easiness to obtain [34].

Currently, solid wastes from different industries like food, beer and wine, agriculture, paper, textiles, detergent, and animal feed industries are used as a substrate for SSF. As a general rule, substrates that remain solid and also contain low moisture levels are the ones that are most preferred for this type of biotechnology process.

However, and despite looking for the almost absence of water, the solid substrate must contain enough water to support the growth and metabolism of microorganisms [4]. In fact, low moisture levels, may lead to poor accessibility of nutrients and a lower degree of substrate swelling, resulting in poor microbial growth and decreased enzyme production. On the other hand, higher moisture contents appeared to cause decreased porosity, loss of particle structure and development of stickiness, which, in turn, prevented oxygen penetration [35].

In processes involving SSF pre-treatments steps may be involved in order to improve the availability of nutrients and reducing the size of its components by processing it mechanically, chemically, or enzymatically [4].

Physico-chemical and environmental factors are possible to be exchanged in order to obtain different results and meet the intended expectations. Type of inoculum, moisture, pH, temperature, time of inoculation, type of substrate/substrate proportions, particle size, aeration/agitation and nutritional factors are some of the aspects most commonly study and reviewed in literature. [4], [36].

During the last decade, SSF has emerged as a potential technology to produce microbial derived products such as feed, fuel, food, industrial chemicals, and pharmaceutical products, and it has shown great potential of application in biorefinery processes producing added valued products from agriculture and food industry raw materials. Being, in this way, an ecologically viable alternative to conventional, traditional and more pollutants physical and chemical treatments of these wastes [20], [37].

This process, when compared with the submerged fermentation (the more traditional one) presents many advantages, for example: this process is more cost-effective, eco-friendly and it takes less

energy and time to be accomplish (sterilization and agitation). It produces less wastewater with less risk of bacterial contamination and with higher titers of compounds produced. Thus, the downstream processes are facilitated and less expensive. Lastly, the degradation of enzymes by undesirable proteases is, in comparison, also minimized [34], [38], [39].

On the other hand, there are also some disadvantages when compared with submerged fermentation. The first and perhaps the most notable, the engineering problems possible to appear by increase of temperature, oxygenation, heat, and mass transfer. Other difficulties are related to pH control; substrate, moisture gradients and non-uniformity of the cell mass. In addition, it is also possible to observe and verify that processes like steady aeration and biomass measurement for microbial growth are more difficult to be accomplish in SSF, just like the study of growth and kinetics. [34], [38], [39].

Currently, SSF is already a commonly studied "green biotechnological" process with high interest and potential for the scientific community. However, more studies and more scientific advances are needed, especially in the use of this process on a larger scale with suitable bioreactors and with a greater diversity of microorganisms [34].

1.2.1 Microorganisms used in SSF

As already mentioned, several factors and variables must be considered before any process of SSF in order to obtain the maximum success. One of these factors, is the type of microorganism used.

The microorganisms used in these biotechnological processes can occur as single pure cultures, mixed identifiable cultures or a consortium of mixed indigenous microorganisms [4]. Nonetheless, in order to maximize the production yield, the microorganisms selected should be able to growth at low humidity and have the ability to synthesize large quantities of the desire product per biomass of microorganism [4].

Filamentous fungi are the most suitable microorganism for SSF since this process mimics their natural habitat, allowing the fungi to synthesize considerable amounts of enzymes and other metabolites [4], [40].

In addition, filamentous fungi are good degraders of lignocellulosic materials because they release lignocellulolytic enzymes and exhibit hyphal penetration into the solid substrate. Furthermore, fungal enzymes release phenolic antioxidant compounds and break down the hemicellulose-cellulose matrix, thus releasing fermentable sugars which favors fungal growth [41]–[43]. Besides, the aerobic fungi, especially, are recognized for their high rates of growth and protein secretion [24], [44].

Nowadays, most of the commercialized enzymes (specially lignocellulolytic) are produced by filamentous fungi of the genera *Trichoderma* and *Aspergillus* [45]–[47]. Under SSF, fungi have also proven to be able to produce multiple enzymes with different applications, including detergent manufacturing, food processing, textile and pharmaceutical industries, medical therapy, molecular biology, bioremediation, and biological control [48]. In this process the most commonly used types of fungi are the *Aspergillus, Penicillium* and *Rhizopus.* [49].

Bacteria and yeasts can also be used in SSF, these two other types of microorganisms require comparatively higher moisture content for efficient fermentation, also having a lower yield of production. However due to their high resistance to extreme conditions and capacity of growing in low water activity environment they are worth to be explored in this type of works [24], [33], [50].

The bacteria are mainly of the genus *Bacillus*, specifically its species *subtilis, licheniformis, pumilus* and *firmus* and have been used in production of diverse enzymes [51]–[54]. As for yeasts, *Yarrowia lipolytica* is the one that attracts the most attention, especially for lipase production [55], [56].

Despite this information, the type of microorganisms selected, and strain used depends on the pretended target product and the composition of the solid substrate to be used in the SSF [57].

In this work, two types of microorganism, a yeast and a fungus, were used under SSF with olive pomace, sunflower and rapeseed cakes as their substrates.

The first one, *Candida cylindracea* a non-ascosporic, often unicellular, non-pathogenic and recognized as GRAS (generally regarded as safe) [58]. It is a species not much studied so far in SSF processes but with very promising results in the works carried out in SmF, especially regarding lipase production. This yeast, is in fact, considered as one of the best producers of this enzyme and is commonly used in research owing to its high activity in hydrolytic reactions as well as synthetic chemistry [59].

And the second one, *Trichoderma harzianum,* a species of fungus widespread in soil, decaying wood and vegetable matter, that has recently emerged as a potent producer of hydrolytic enzymes, due its capability of using a wide range of compounds as carbon and nitrogen sources and secrete a variety of enzymes. Apart from this quality as enzyme producer, this genus is of great importance in biological control [60], [61].

A compilation of studies where both of this species were used, whether for enzyme production or other scientific novelty or improvement is presented in the following table (**Table 2**) [58], [60]–[68].

Table 2. Compilation of studies where *Candida cylindracea* or *Trichoderma harzianum* were used with a relevant interest.

Microorganism	Use	Author
Candida cylindracea	Production of lipase using a fed-batch fermentation and olive-mill wastewaters	Gonçalves *et al.* [62]
Candida cylindracea	Biological treatment of olive-mill wastewater	Gonçalves *et al.* [63]
Candida cylindracea	Lipase production and olive-mill wastewater valorization	D'Annibale *et al.* [64]
Candida cylindracea	Optimization of lipase production in palm oil-mill effluent based medium	Salihu *et al.* [58]
Candida cylindracea	Assessment of olive-mill wastewater as a growth medium for lipase production	Brozzoli *et al.* [65]
Trichoderma harzianum	Production of lipase by SSF	Toscano *et al.* [60]
Trichoderma harzianum	Comparison of SmF and SSF of agro-industrial residues for the production and characterization of lipase	Coradi *et al.* [66]
Trichoderma harzianum	Production and characterisation of lignocellulolytic enzymes from SSF of rice straw	Rahnama *et al.* [67]
Trichoderma harzianum	Biologic control and elicitor of defense response in chickpea	Sreeramulu *et al.* [61]
Trichoderma harzianum	Production of enzymes in SSF of citrus peels	Nabi *et al.* [68]

1.2.2 Applications

The final components obtained in SSF may have different interests and applications depending on solid substrate used, the type of microorganisms selected and the intentions with which they are produced.

In order to separate the final products obtained in the process, they must be firstly exposed to a series of downstream processes: the downstream process consists of the obtention of crude extract with a filtration step to remove the fermented solid, ultrafiltration to remove the microorganism, and concentration of the product and lyophilization to obtain the enzyme or other added-value products as a dry solid [69].

Nowadays, the SSF process is used in divergent areas, benefiting of its advantages and presenting useful novelties for the different industries:

For the production of second-generation biofuels by hydrolyzing hemicellulose and cellulose to simple sugars and, subsequently, using these sugars to obtain bioethanol [70], [71], also producing biofertilizers that influence the soil ecosystem and improve the fertility of the terrain [72]–[74], as well as for the production of microbial biomass in order to recover proteins and food products for human consumption (Mushrooms, preservatives and flavoring/coloring agents) [75]–[79]. Besides that, other uses, more related to the pharmaceutical industry like antibiotics, nutraceutical and therapeutical compounds but also cosmetics have also been tried out [80], [81], helping to reduce their production cost, thus making them cheaper on the market for the consumer.

Besides the bioproducts mentioned so far, the one that is mostly produced with this low-cost biotechnological technique and is more studied for the valorization of industrial agroproducts wastes are enzymes. These compounds are naturally produced and secreted by the microorganisms during their growth in the solid substrate and after the fermentation process occurs, need to be recovered and separated of the microorganisms, centrifugation for extracellularly produced enzymes or lysing of cells for intracellular enzymes and the most common methods [49], [82].

If currently, most industrial enzymes in the market are produced by microbial cultivation under SmF, often using genetically modified microorganisms [49], new solutions, using SSF, has been implemented in order to reduce the costs involved and enhance the field of application, especially if residues are used as substrates [25], [38], [83].

Under SSF, varied enzymes have already been produced with different applications, including, detergent manufacturing, food processing, textile and pharmaceutical industries, medical therapy, molecular biology, bioremediation, and biological control [22].

Lipases, proteases, cellulases, xylanases, β-glucosidase and phytase are some examples of the enzymes produced with SSF and are the ones that were assessed in this work.

A compilation of some recent works where enzymes were produced through SSF is presented in the table below (**Table 3**) [6], [22], [31], [84]–[88].

Table 3. Some recent examples of the numerous studies and works compilation where it has been produced enzymes through SSF using agro-industrial waste as a substrate.

Enzyme(s) produced	Microorganism(s)	Substrate(s)	Author
Lipase	*Yarrowia lipolytica*	Olive pomace and wheat bran	Lopes *et al.* [84]
Lipase	*Yarrowia lipolytica*	Canola and soybean cake	Souza *et al.* [85]
Lipase	*Aspergillus oryzae*	Castor de-oiled cake	Jain *et al.* [86]
Lipase	*Penicillium roqueforti*	Cocoa shells	Sales de Menezes *et al.* [87]
Lignocellulolytic enzymes	*A. ibericus, A. niger & R. oryzae*	Wineries, olive mill and brewery wastes	Leite *et al.* [31]
Lignocellulolytic enzymes	*Aspergillus ibericus*	Winery and olive mill wastes	Filipe *et al.* [22]
Lignocellulolytic enzymes and protease	*A. ibericus, A. niger & R. oryzae*	Sunflower, rapeseed and soybean cake	Sousa *et al.* [6]
Phytase	*A. niger & ficuum*	Wheat bran, rice bran, and groundnut cake	Shivanna *et al*. [88]

In addition to all these bioproducts obtained and mention, SSF have also proved to contribute to the biotransformation and enrichment of the agroindustrial substrate itself. Helping to reduce or eliminate some toxins present and upgrading, as well, their protein value and digestibility [1], [23], [89]–[92]

These enriched fermented substrates obtained by SSF can be used as animal feed in the agricultural sector (e.g., livestock and aquaculture). Furthermore, it provides economic and social assistance to the

world, reducing the excess of sub-products with low value and partially preventing environmental pollution [1]

2. Objectives

The main objective of the work is to add value to the pomace that results from the production of olive oil, the olive pomace or olive cake, since is one of the most unexplored vegetable oil cakes. In order to achieve this end and produce value-added products, namely enzymes, such as lipase, protease, cellulase, xylanase, β-glucosidase, and phytase, that can nutritionally improve the substrate allowing it to be used in animal feed, this substrate will be mixed with other two agro-industrial by-products of vegetable oil production, sunflower cake and rapeseed cake, in a green biotechnological process referred as SSF and using two different microorganisms, *Candida cylindracea* and *Trichoderma harzianum.*

Different conditions of SSF will be tested, varying different factors (mixed substrate composition, time of fermentation and moisture) with the purpose of achieving optimal settings and maximum enzymatic activity values of extracellular enzymes produced as possible.

It is expected that this project will confirm SSF potential and help in the valorization of agro-industrial wastes, especially olive pomace. Giving it an alternative second life and a viable and sustainable purpose in a circular economic context, and like this, assisting in the combat of the many environmental problems associated with this vegetable oil cake.

3. Materials and methods

3.1. Substrates

Olive pomace was obtained from a local two-phase olive mill plant in the north region of Portugal (e.g., Amarante and Mirandela) in the season of 2017/2018 and stored at −20 °C with the need to be previously thawed and kept in the fridge whenever they were used throughout the study. Sunflower cake and the rapeseed cake were supplied in 2018 by Iberian companies (Pargasor group, J.C. Coimbra ii – Distribuição S.A., Iberoils - Sociedade Ibérica de Oleaginosas and Sorgal - Sociedade de óleos e rações, S.A.). These last two by-products were stored in bags and conserved at room temperature.

The characterization of the agroindustrial by-products used in this work in presented in **table 4.** This characterization was carried out by other collaborators and students according to the protocols commonly used in the LBB - Laboratory of bioprocesses and biosystems of University of Minho.[6], [31]

Table 4. Characterization ff the vegetable oil cake used in this work.

Substrate composition (%)	Oil cakes		
	OP	RSC	SFC
Lipids	10.5 ± 0.9	2 ± 0	1.3 ± 0.0
Crude protein	3.8 ± 0.3	39.8 ± 2.1	39.7 ± 0.6
Carbon	51.3 ± 0.7	50.6 ± 0.8	46.7 ± 0.2
Nitrogen	0.6 ± 0.0	6.3 ± 0.3	6.4 ± 0.1
Cellulose	10 ± 0	15.5 ± 1.1	11.1 ± 1.3
Hemicellulose	18 ± 2	13.6 ± 1.0	12.6 ± 0.5
Lignin	44.6 ± 0.3	8.7 ± 2.7	6.6 ± 0.7
Reducing sugars	7 ± 0	1 ± 0	1 ± 0
Total phenols	0.6 ± 0,0	0.5 ± 0,0	1 ± 0
Soluble protein	0.1 ± 0,0	1 ± 0	1 ± 0

3.2. Biological material

Candida cylindracea CBS (7869) and *Trichoderma harzianum* (2413) (MUM culture collection, Braga, Portugal) were the microorganisms used in this assay.

The yeast, initially stored at -80°C, was inoculated into an extract peptone dextrose agar medium plate (YPDA) and stored at the fridge at 4 °C. This first plate worked as stock and in order to keep this microorganism fresh and viable, every two weeks new *C. cylindracea* plates in YPDA medium were inoculated using biomass scrapings from the first plate. The YPDA medium had the following composition: peptone 20 g/L, glucose 20 g/L, yeast extract 10 g/L and agar 20 g/L.

On the other hand, the fungus is preserved in glycerol stocks stored at -80 °C. From there cultures were made on potato dextrose agar (PDA) plates (15 g/L agar, 20 g/L dextrose, 4 g/L potato extract),

and subcultured in PDA slants, which were incubated at 28 °C for 7 days. These slants were stored at 4 °C and the spores present in the slant used as inoculums for SSF.

3.3. Solid-state fermentation

Solid state fermentation (SSF) was carried out in 500 mL Erlenmeyer with 10 g of dried solid substrate. Moisture levels (wet basis) and substrate proportions were controlled and changed as desired. Erlenmeyers with solid medium were sterilized at 121 °C for 15 min.

Yeast inoculation was performed using a pre-inoculum where the yeast cells were allowed to grow overnight in YPD medium (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L). After this overnight grow at 27°C and 200 rpm, the medium was centrifuged (10 min at 5000 rpm) and the cells resuspended in sterile 0.9% (w/v) NaCl. The OD was adjusted according to the calibration curve constructed previously so that the initial biomass was around 10^7 cel/g of dry solid in SSF experiments.

For the inoculation of the fungi into the substrate mixture, an autoclaved (121 °C for 15 min) peptone solution composed of 0.1% (w/v) peptone and 0.01% (w/v) Tween-80 was added into the PDA slants and the spore concentration was then adjusted to 10^6 spores/mL using a Neubauer counting chamber. 2 mL of the spore solution was inoculated into each erlenmeyer.

All flasks were then maintained at controlled temperatures (28°C for the fungus and 27°C for the yeast) variating the time in the incubators as pretended.

3.4. Extraction of fermented oil cakes

Compounds produced during SSF were recovered using a solution composed of 1% (w/v) NaCl and 0.5% (w/v) Triton-X100 and stirring at 150 rpm for 30 min in an incubator at room temperature. Following that, the mixture (fermented oil cakes and solution) were filtered through a fine-mesh net to a falcon tube. 1 ml of the filtrate was used to counter the number of cells per Neubauer counting chamber at the microscope and the rest centrifuged at 8000 rpm for 10 minutes at 4 °C, the resulting supernatant was stored in Eppendorf tubes at −20 °C until further analysis.

3.5. Analytical methods

Reducing sugars (RS) were measured by the DNS method and a calibration curve made with glucose [93]. Briefly, 100 µL of DNS (3,5-dinitrosalicylic acid) were mixed with 100 µL of each sample (100 µL of H_2O for the blank). If necessary, the samples are diluted in distilled water. After that, the tubes were placed in a boiling water bath for 5 min and cooled in a cold water bath where 1 ml of distilled water was added. After reaching room temperature the absorbance was read at 540 nm on a microplate.

Soluble protein (SP) concentration in the extracts was determined by the method of Bradford using bovine serum albumin as standard [94]. In this protocol, 10 µL of sample (H_2O for blank) were added in each well of a microplate to 300 µL of Comassie blue. The microplate was then shaken for 30 seconds and left to stand in the dark at room temperature for 10 min. After that, the absorbance was read at 595 nm.

Lipase activity was determined by a spectrophotometric method, using a reaction mixture composed of 20 µL of enzymatic extract with 980 µL of 2.63 mM p-nitrophenyl butyrate in sodium acetate buffer 50 mM pH=5.6, prepared just before the start of the assay. The tubes, covered in aluminum, were placed in a bath at 37 °C for 15 min and 2 mL of acetone were added in order to stop the reaction, the absorbance was read at 405 nm. In order that the color of the sample did not interfere with the absorbance value read, color controls (blanks) were carried out, where the sample with the enzymatic extract was added only after the reaction had been stopped with the acetone. One unit of lipase activity (U) was expressed as the amount of enzyme which produces 1 µmol of p-nitrophenol per minute, under the assay conditions.

Protease activity was quantified by adding the following in a 2 mL Eppendorf tube: 0,5 mL of enzyme extract and 0,5 mL of azocasein substrate 0,5% (w/v) in acetate buffer at pH 5.0. The Eppendorf's were incubated at 37 °C for 40 min and after this time 1 mL of 10% (w/v) trichloroacetic acid was added in order to stop the reaction. The tubes were then subsequently centrifuged (3000 rpm, 15 min). To the recovered supernatant was added 1 ml of potassium hydroxide solution (KOH 5M) in test tubes and the absorbance was read at 428 nm on a microplate. Color controls (blanks) were also carried out during the assay, adding the enzyme extract only after the reaction has been stopped. One unit of activity was defined as the amount of enzyme per minute that causes an increase of 0.01 of absorbance, relative to the blank, under assay conditions

For xylanase determination, 250 µL of beechwood xylan solution (1% (w/v) in citrate buffer 0.05 N at a pH of 4.8) and 250 µL of diluted sample were added to the test tubes (for the blank, 250 µL of citrate buffer 0.05 N at pH 4.8 was used instead of the diluted sample), the test tubes were then heated in a bath at 50 °C for 15 min. Thereafter, 500 µL of DNS was added to the mixture and the tubes were placed in a bath at 100 °C for 5 minutes; after cooling to room temperature, 5 ml of distilled water was added, and the absorbance was read at 540 nm by a microplate reader.

For cellulase quantification, the process was the same as the above described for xylanase except for the substrate used, which was 2% (w/v) carboxymethylcellulose in citrate buffer 0.05 N at a pH of 4.8 and the incubation time, 30 min instead of 15. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 µmol of glucose or xylose per minute under standard assay conditions for cellulase and xylanase, respectively.

β-glucosidase activity was determined using 100 µL of 4 mM, 4-nitrophenyl β-D-glucopyranoside (PNG) as substrate in citrate buffer (50 mM, pH 4,8) and 100 µL of diluted sample. The enzymatic reaction was carried out at 50 °C for 15 min and the p-nitrophenol liberated was measured at 400 nm, after adding 600 µL of Na_2CO_3 (1M) and 1.7 mL of distilled water. Enzyme activity was defined as the quantity of enzyme required to release 1 µmol of p-nitrophenol per minute under standard assay conditions.

The phytase activity present in the final enzymatic extracted was measured by mixing 0.25 mL of this extract with 0.25 mL of sodium phytate (15 mM) in test tubes and incubating the mixing at 40 °C for 45 minutes. After that, 4 mL of the following solution were added in order to stop the reaction: acetone, ammonium molybdate and sulfuric acid (2:1:1 v/v). 0.4 mL 1 M citric acid were also added subsequently, and the amount of free phosphate was determined by spectrophotometry at 355 nm. One unit of enzyme activity was defined was the amount of enzyme needed to release 1 µmol of phosphate per minute under standard assay conditions.

All enzymatic activity values were expressed in units per gram of dry substrate (U/g).

3.6. Statistical analysis and experimental design

Results are presented as the mean ± standard deviation (SD) of at least two replicates. The statistical analyses were carried out using GraphPad Prism 9. Statistically significant differences of the

several assays tested were evaluated by a one-way or two-way ANOVA and multiple comparison tests. A significant difference was considered if $P < 0.05$ applying the Tukey multiple-comparisons test.

The Box-Benchen experimental design (BBD) was used to find the optimal conditions that maximized enzymatic activity produced by SSF with the fungus *Trichoderma harzianum*.

The assessment were performed with three factors (% of moisture, % of RSC and % of SFC) at three different levels (-1,0,1). Thus, the moisture varied between 65%, 75% and 85% (w/w) on a wet basis in the multiple runs.

Furthermore, the different percentages 0%, 25% and 50% (w/w) of SFC and RSC tested in the experimental design were always complemented with the remaining % of OP (0%,25%,50%,75% or 100% (w/w) depending on the run), in order to reach the logical 100% of substrate mixture.

Besides, incubation time and temperature were kept constant at 7 days. 15 experimental runs were conducted with three replications at the central point. The response surface analysis was based on a multiple linear regression that considered the main, quadratic, and interactional effects. The reliability of the regression model was evaluated by using the coefficient of determination (R^2) and the analysis of variance (ANOVA). For each variable, the quadratic models were represented as 3D response surface plots and Pareto charts using the Statgraphic software.

4. Results and discussion

4.1. *Candida cylindracea*

4.1.1 Effect of the different solid substrates in SSF

The composition of the solid substrate is one of the most important factors for the growth of microorganisms in SSF. Initially and in order to verify the capacity of these three agroindustrial wastes to serve as substrate with the yeast *Candida cylindracea,* SSF processes were carried out during a period of four days using the substrates without any mixtures.

This capacity was evaluated by measuring yeast growth, the concentration of reducing sugars (RS), soluble protein (SP) and enzymatic activity, more specifically, lipase and protease.

In lipase enzyme activity (**Fig. 1 A**), the results vary according to the substrate used, with the fermentation using only SFC and the fermentation using only RSC, producing lipolytic activity values statistically different of 55.5 ± 2.5 U/g and 35.2 ± 2.5 U/g, respectively. ($P < 0.05$). Additionally, it is also possible to observe that the yeast using the OP by-product as substrate does not have the capacity to produce lipase, since at the end of the four days the enzymatic activity is null.

In the production of the protease by the *Candida* (**Fig. 1 B**)*,* all three conditions are capable of producing the enzyme. However, the substrate with only OP, has, once again, manifested the lowest activity values (19.8 ± 2.6 U/g), followed by the RSC (33.1 ± 6.3 U/g) and lastly, with the highest activity values, the SFC (54.8 ± 9.6 U/g).

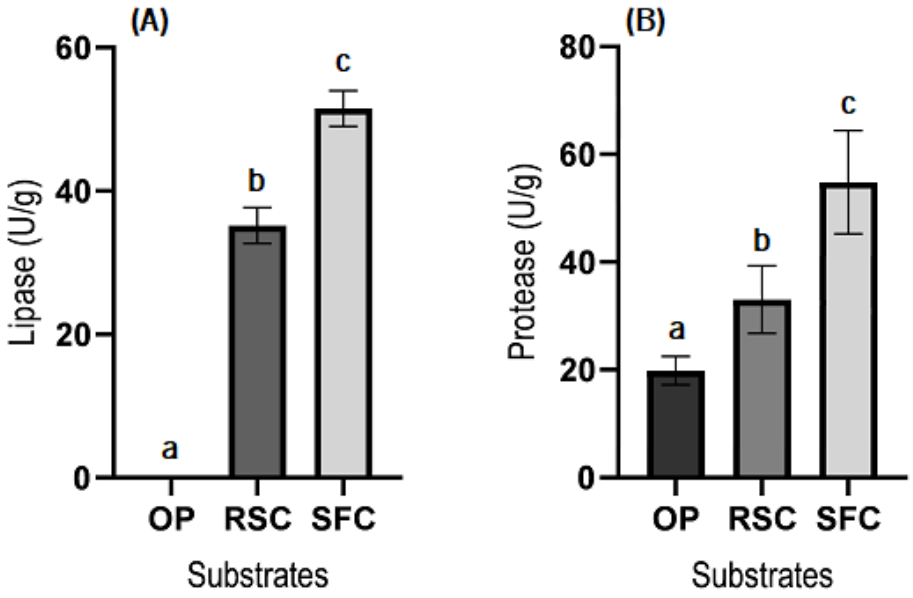

Figure 1. Lipase (A) and Protease (B) activity (U/g) on SSF with *C. cylindracea* over 4 days using different substrates - Olive pomace (OP), sunflower cake (SFC) and Rapeseed cake (RSC). Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters are not significantly different.

In **figure 2 A**, the results regarding biomass growth also showed a favoring using SFC and RSC when compared with OP, as it would be expected, considering the characterization and composition of the substrates (**Table 4**), mainly at the level of protein and nitrogen, important elements concerning microbial growth. This inability of the yeast to grow under the OP substrate ends up influencing the results obtained for the enzymatic activities under the same circumstance. (**Fig.1**)

Assessing the consumption of RS over the 4 days of SSF (**Fig. 2 B**), only the OP substrate did not reveal the capacity to consume the sugars, which in turn helps to explain the mediocre growth observed in **figure 2 A**, where it was divulged their incapacity to growth. Relatively to the other single substrates, they consumed approximately the same concentration of sugars, around 30 mg/g of solid substrate.

With regard to SP variation (**Fig. 2 C**), it was noticed higher variation (9.7 ± 1.4 mg/g and 5.7 ± 1.4 mg/g) in the trials with SFC and RSC, respectively, when compared to the one with OP as an individual solid substrate, that even showed negative variation of concentration at the end of the 4 days of SSF, possibly explained by the consumption of SP by the yeast throughout the fermentation period in an attempt to growth under very stressful condition.

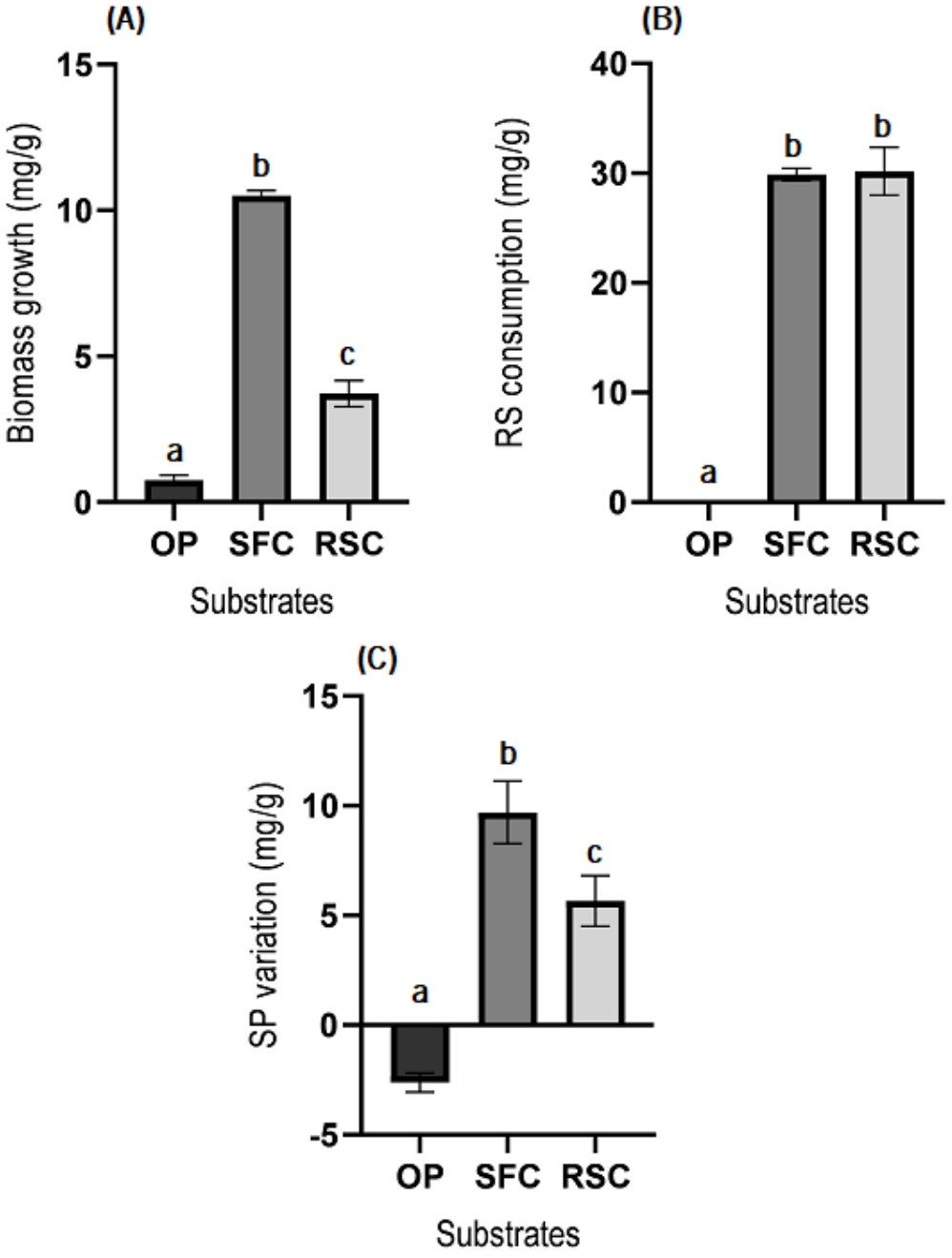

Figure 2. Effects of SSF with *C. cylindracea* and an incubation time of 4 days using different substrates - Olive pomace (OP), sunflower cake (SFC) and Rapeseed cake (RSC) - on biomass growth (A), reducing sugars consumption (B) and soluble protein variation (C) (mg/g). In order to achieve values that represented the variation over the 4 days, in all concentrations was subtracted the concentration already present in the beginning of the SSF process. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

Considering the results observed in **Figure 1 and 2** and the main objective of valuing the OP and producing value-added products using this agro-industrial substrate, it is verified the need to mix this by-product with others, more capable and with better results (RSC and SFC, in this case), since individually, OP does not provide the necessary conditions to this microorganism to growth (**Fig.2**) and produce any goods (**Fig. 1**), proving once again to be a by-product that is difficult to value and treat.

4.1.2 Effect of the different solid substrates mixtures in SSF

The use of a single solid agro-industrial by-product as a substrate sometimes may not be enough to provide the growth of yeasts and fungi, since it may not provide the necessary nutrients for microorganisms growth [95]. Olive pomace (OP) proved to be an example of one of these substrates in the previous results (**Fig.1 and 2**), where it did not provide the necessary conditions to the yeast *Candida cylindracea,* largely due the lack of nitrogen and protein. Thus, there is a need to mix it with other by-products of the same origin in order to balance the nutritional composition of the medium and proceed to its valorization and production of value added novelties.

Likewise, OP was mixed with rapeseed cake (RSC) at 3 different mass proportions of 1:2, 1:1 and 2:1 of RSC+OP (corresponding to a percentage of 33%, 50 % and 66% (w/w) of RSC) and SSF processes were carried using the yeast *C. cylindracea*.

As previously the results for the isolated OP substrate showed weak enzymatic activity at the end of 4 days (null regarding lipase and inferior to the other substrates regarding protease), in these new fermentations using the mixtures, the possibility of a peak in enzyme activity being observed before the fourth day was also studied. Therefore, and in addition to a 4-day fermentation period, SSF were also carried out by two days.

Results on enzymes (lipase and protease measured in U/g), obtained by SSF are depicted in **Figure 3**, as well as biomass concentration and nutrient solubilization (SP and RS measured in mg/g of solid substrate) available in **figure 4 and 5**.

Lower values and practically constant (not far from null) values of lipase activity were observed with 50% and 33% of RSC (**Fig. 3 A**). However, an increase in this enzyme activity was detected when 66% of RSC (2:1 RSC+OP) was used as the solid substrate, reaching a 6.3 ± 1.3 U/g peak at the end of fermentation. Either at second or fourth day, the production of this enzyme is significantly higher when compared to the other ratios ($P < 0.05$).

Regarding protease (**Fig.3 B**), a general increase over time in its activity was detected in all different substrate mixtures used, and, once again, the higher value was achieved when using the substrate with 66% RSC. However, it is not possible to verify significant differences between the mixes after 4 days of fermentation. Nevertheless, at the second day it is possible to observe that the ratio with less RSC is significantly lower than the others. At the end of the SSF process, this same proportion, is also the one that obtains the lowest activity values, but again, not in a significantly way ($P < 0.05$).

In both cases, concerning the two enzymes, the initial activity value is 0 at the beginning of SSF, indicating that, when fermentation starts the enzymes are yet not present in the environment, revealing that, in fact, there is enzyme production during the SSF with the yeast.

Comparing the activity values of the two enzymes, it is possible to verify a greater production of protease than lipase, under the same conditions of time and substrate ratio. Furthermore, the results obtained in **figure 3** prove what has already been observed previously, where substrates with a higher RSC ratio obtain higher values of enzymatic activity, corroborating the difficulty of using OP alone in SSF.

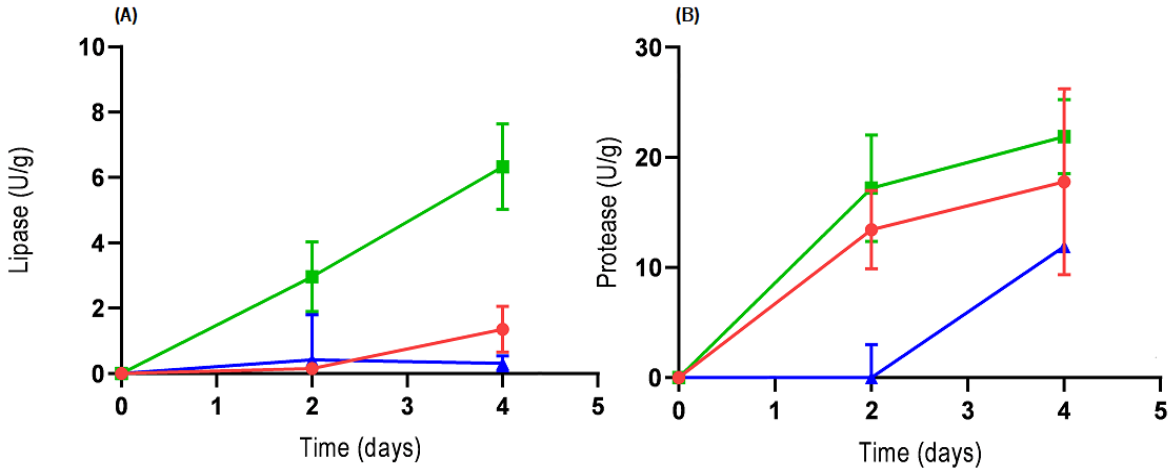

Figure 3. Effect of substrate mixture of rapeseed cake (RSC) and olive pomace (OP) in different mass proportions: 66% RSC (green line), 50% RSC (red line) and 33% RSC (blue line) on the activity of lipase (A) and protease (B) (U/g) over 4 days of SSF with *C. cylindracea.*

Figure 4 compares the different substrate ratios concerning concentration of biomass and concentration of RS. Regarding biomass growth, the different substrates reveal to be significatively different from each other ($P < 0.05$). Consequently, it is possible to perceive a higher final biomass concentration with the substrate with more RSC, reaching a concentration of 32.4 ± 0.9 mg/g at the end of the SSF process. Additionally, the fact that this mixture is the one that shows better growth helps to explain the higher enzymatic activity also observed using this proportion, for lipase and for protease (**Fig.3**).

On the other hand, if we consider the concentration of RS (**Fig. 4**), the different substrate mixtures do not present significant differences when compared between themselves, except for the mixture with 66% RSC in the beginning of the SSF, that is significantly smaller to the rest in this same period ($P <$

0.05), indicating that when the SSF starts there are less available sugars in this substrate when compared to the remains.

Furthermore, still in **figure 4**, comparing the biomass concentration over time with the concentration of RS in the 4 days of SSF, it is possible to verify that, as expected, an increase in biomass concentration is related and accompanies a decrease in RS, regardless of the substrate mixture used. This phenom is explained by the consumption of reducing sugars during the growth of the yeast. In this way, the substrate mixture with more percentage of RSC seems to be more efficient than its peers since it grew the most consuming less sugars.

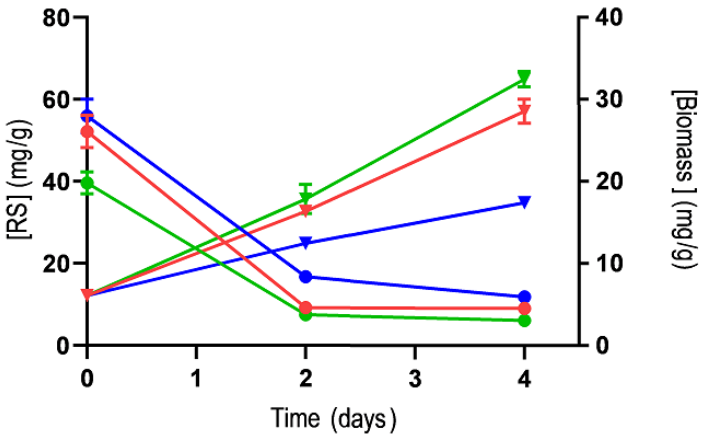

Figure 4. Effect of substrate mixture of rapeseed cake (RSC) and olive pomace (OP) in different mass proportions: 66% RSC (green line), 50% RSC (red line) and 33% RSC (blue line) on the concentration of reducing sugars (RS) "dots" and concentration of biomass "triangles" (mg/g) over 4 days of SSF with *C. cylindracea.*

In the **Figure 5**, it is possible to perceive a general increase in the concentration of soluble protein in the different proportions of substrate, over time, in all samples, during the SSF period. However, the difference between the different proportions is not statistically significant according to a Tukey´s multiple comparison test ($P < 0,05$).

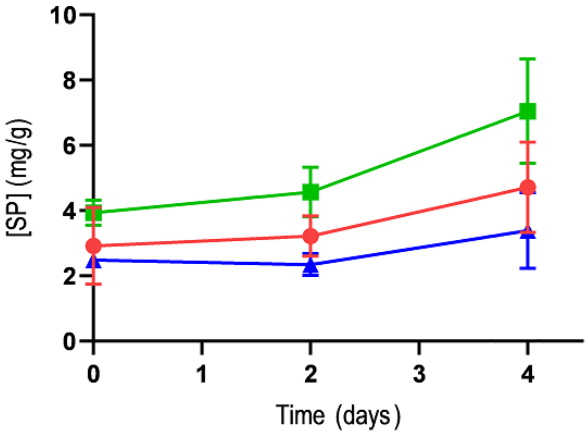

Figure 5. Effect of substrate mixture of rapeseed cake (RSC) and olive pomace (OP) in different mass proportions: 66% RSC (green line), 50% RSC (red line) and 33% RSC (blue line) on the concentration of soluble protein (SP) (mg/g) over 4 days of SSF with *C. cylindracea.*

With the results presented it is possible to conclude that the most favorable substrate proportion was the one with more percentage of RSC (66%) (RSC+OP 2:1), since it was detected statistically significant higher growth (**Fig. 4**) and lipase activity (**Fig. 1 A**) when compared to the other substrate proportions. In the remaining concentrations and activities, this same proportion also obtains the best results, although in a statistically non-significant way.

The results are in line with expectations since this particular mixture (RSC 66% + OP 33%) is the one with the highest proportions of RSC, a lignocellulosic substrate that, as already said, when compared to the OP substrate (**Table 4**), presents higher levels of available crude protein and nitrogen, which makes it the best fit for microbial growth, since crude protein and N are very important parameters to be considered when judging if a substrate is more or less suitable to be a substrate in microorganism growth.

4.1.3 Solid-state fermentation kinetics

Fermentation time is another important parameter when stablishing a SSF process. Since in previous shown experiments, enzymes production increase with fermentation time, this parameter was further increased till 8 days of SSF in order to analyze its impact on enzymes production, nutrient solubilization and biomass growth, using the previous best substrate mixture of 66% RSC and 33% OP (w/w).

In lipase activity (**Fig 6 A**) it is possible to glimpse that the activity increased in the first days of fermentation, reaching a peak at the fourth day (6.3 ± 1.3 U/g). After this point, this enzyme activity decreased, significantly, until the end of the SSF process ($P < 0,05$). Demonstrating that the increase in fermentation time period is not translated in the increase of lipase production by the yeast in the SSF.

Similarly, Moftah *et al.,* 2012 studied the production of this enzyme using *Candida utilis* and olive pomace as a solid substrate and reported maximum activity, after 4 days, of approximately 25 U/g [35]. *C. parapsilosis* was also used in the work of Kannoju *et al.*,2017 using *Jatropha* de-oiled seed cake as substrate and the optimum time reported was also of 4 days [96]. Moreover, Rekha *et al.*,2012 reported a maximum activity after 5 days of fermentation using *Candida rugosa* and groundnut oilcake as solid substrate [97]. In addition , other yeasts have also been used for SSF and lipase production, and usually, the optimal time associated with the production of this enzyme is also around 4/5 days. [98]

On the other hand, for protease (**Fig. 6 B**), maximum activity was observed after 6 days of fermentation (24.5 ± 1.5 U/g). However, the differences between the protease activity values in the fourth and sixth day are not statistically significant. Likewise, it appears that extending the SSF process also had no significant effect in the production of proteolytic enzymes.

Comparing with other works where this enzyme was also produced, Moftah *et al* ., 2012 obtained a maximum activity value of 58 U/g after 5 days of fermentation using *C.utilis* and agroindustrial waste as a solid substrate [35].

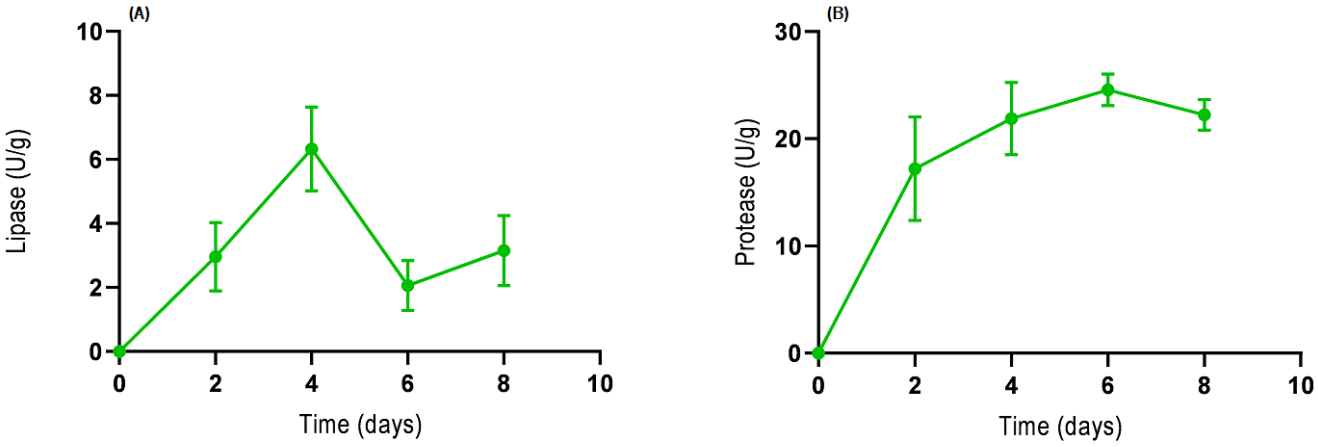

Figure 6. Effect of SSF time in lipase (A) and protease (B) production by *C. cylindracea* using RSC+OP (2:1) as substrate.

Overall, and regarding both enzymes, it can be concluded that the results obtained are in congruence with the ones already reported, demonstrating that the production of lipase reaches its maximum after 4 or 5 days of fermentation and ceases after the exponential cell growth, decreasing rapidly. The decay of this extracellular enzyme activity may be related to the effect of pH, temperature, or can be probably caused by proteolysis, similarly to what as proposed by Moftah *et al.* 2012 [35]. This last cause, in this case, seems to be the most convenient explanation, since protease production occurs at higher levels than lipase, and their peak is reached only at the sixth day, it is possible that these proteolytic enzymes produced are perhaps breaking down, into peptides or amino acids, the other enzyme, that, in this way, end up decreasing their activity, as show in the **figure 6**.

Regarding the concentration of biomass and the concentration of RS over time (**Fig. 7**), it is also not possible to observe statistical differences in the new times - sixth and eighth day - and the previous one - day four. Thus, it can be concluded, that after four days of SSF there is no yeast growth and multiplication worth to be noticed, as well as no consumption of RS after the same period of time, this can be associated with a cessation of growth by the yeast.

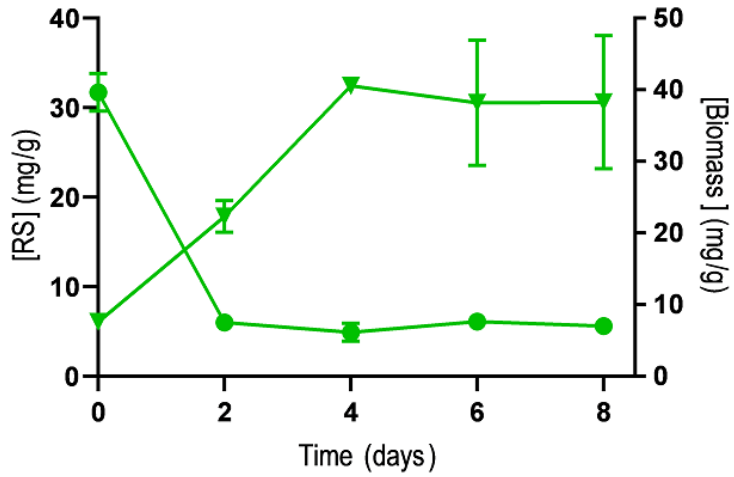

Figure 7. Effect of SSF time in the concentration of reducing sugars (RS) "dots" and concentration of biomass "triangles" of *C. cylindracea* using RSC+OP (2:1) as substrate.

Lastly, for the SP concentration over time (**Fig. 8**), although there is a slightly higher concentration of soluble proteins at the end of the 4th day, this difference is not statistically relevant when compared to the values of the following days. Revealing, once again, that an increase in days is not justified and does not show particularly noticeable differences relative to what has already been observed.

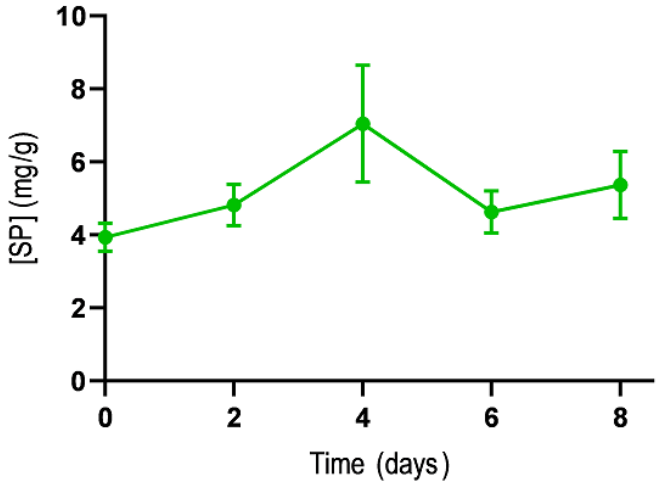

Figure 8. Effect of SSF time in the concentration soluble protein (SP) with *C.cylindracea* using RSC+OP (2:1) as substrate.

After analysis of the effects of SSF time, it is possible to state that the period of 4 days is the optimal time for the production of lipase and protease, as well as nutrient solubilization and biomass growth using RSC and OP as the solid substrate. Regarding protease, despite the peak was reached at the sixth day, there are no significant differences with the activity obtain in the day four, and in this way, there is no necessity to prolong the fermentation more than 4 days.

4.1.4 Moisture effect

After determining the optimal substrate mixture and fermentation time for enzyme production, biomass growth and nutrient solubilization, the effect of the moisture was also evaluated, since moisture levels, as already stated are another important parameter in SSF [35].

As the *C. cylindracea* can be considered a non-conventional yeast and poorly study in SSF processes for the production of value added products, despite having promising results regarding fed-batch fermentation and biological treatment of olive mills wastewater, as reported by Gonçalves *et al.,* 2009 and 2012 and D´Annibale *et al.,* 2006 ([62]–[64]), a wide range of moistures were tested in order to seek the one where it was possible to optimize the nutrient solubilization and biomass growth as well as the lipolytic and proteolytic activities.

Therefore, SSF was carried over 4 days with a mixture of RSC+OP 2:1 (corresponding to a percentage of 66% RSC and 33% OP (w/w)), which was previously proved to be the best condition for lipase and protease production, adjusting the moisture to 65%, 75% and 85% (w/w) on a wet basis, in order to test the influence of this variation.

For lipase production (**Fig. 9 Dark bars**), 75% moisture was confirmed as the best values to obtain the highest activity, with all results obtained regarding different moistures being significantly different from each other, after statistical analysis ($P < 0.05$).

As for protease (**Fig. 9 Light bars**), maximum activity values obtained, corresponded, once again, to the condition of 75% initial moisture. However, regarding this enzyme, the results, are not statistically different from each other ($P < 0.05$.)

In **figure 9** it is also noticeable a favoring in the production of proteolytic enzymes in detriment of lipolytic enzymes, with the first enzyme, similarly to what happened previously, obtaining higher values of activity (U/g). Proving once again, that using these substrates and conditions, the yeast revels to be a better producer of proteolytic enzymes.

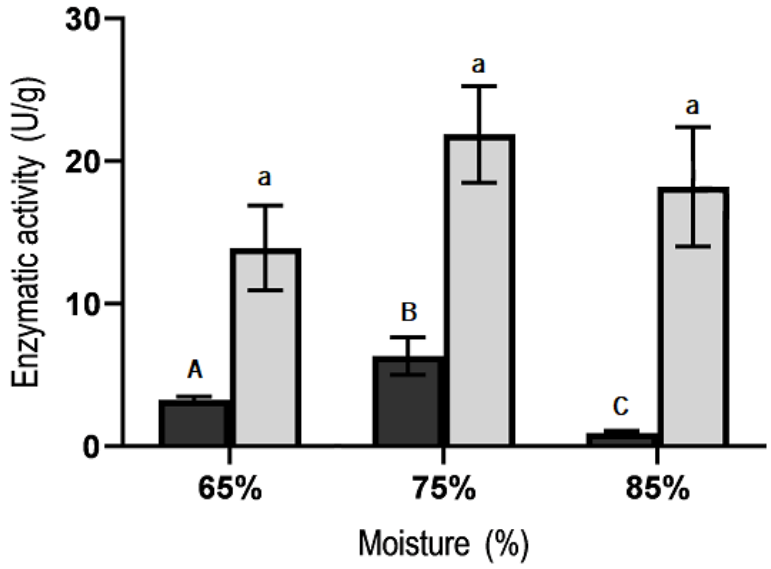

Figure 9. Effect of SSF with *C. cylindracea* using RSC+OP (2:1) as substrate on lipase (dark bars) and protease (light bars) activity (U/g) over 4 days of fermentation using 3 distinct levels of moisture (65%,75% and 85%). Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters are not significantly different.

The results obtained in the **figure 10 A** demonstrate a common increase, as expected, in biomass concentration over the 4 days of yeast inoculation. Biomass growth corresponds to the difference between the concentration present after the four days and biomass already present at the begging of the fermentation, with 85% moisture being the one where it is possible to observe a greater and statistically significant increase reaching a value of 57.5 ± 6.2 mg/g, statistically different from the remains ($P < 0.05$).

In **figure 10 B** It is also possible to observe a generalized consumption of reducing sugars over the four days of SSF. All levels of moisture demonstrate identical consumption of reducing sugars, thus being statistically similar, without any noticeable differences between them ($P < 0.05$). The respective values fluctuate between the 32 and 34 mg/g.

Observing the variation in the concentration of SP (**Fig. 10 C**) (difference between the concentration of SP at day 4 and already present in the beginning of SSF) it is possible to find out a decrease, in the 65% moisture samples of 1.3 ± 0.5 mg/g, and an increase of 4.3 ± 0.8 mg/g, and 1.1 ± 0.7 mg/g on the two subsequent moistures, respectively. With all results being significantly different from each other ($P < 0.05$). The protein decreased under 65% moisture conditions may indicate consumption by the yeast in order to support its growth in extreme stress conditions.

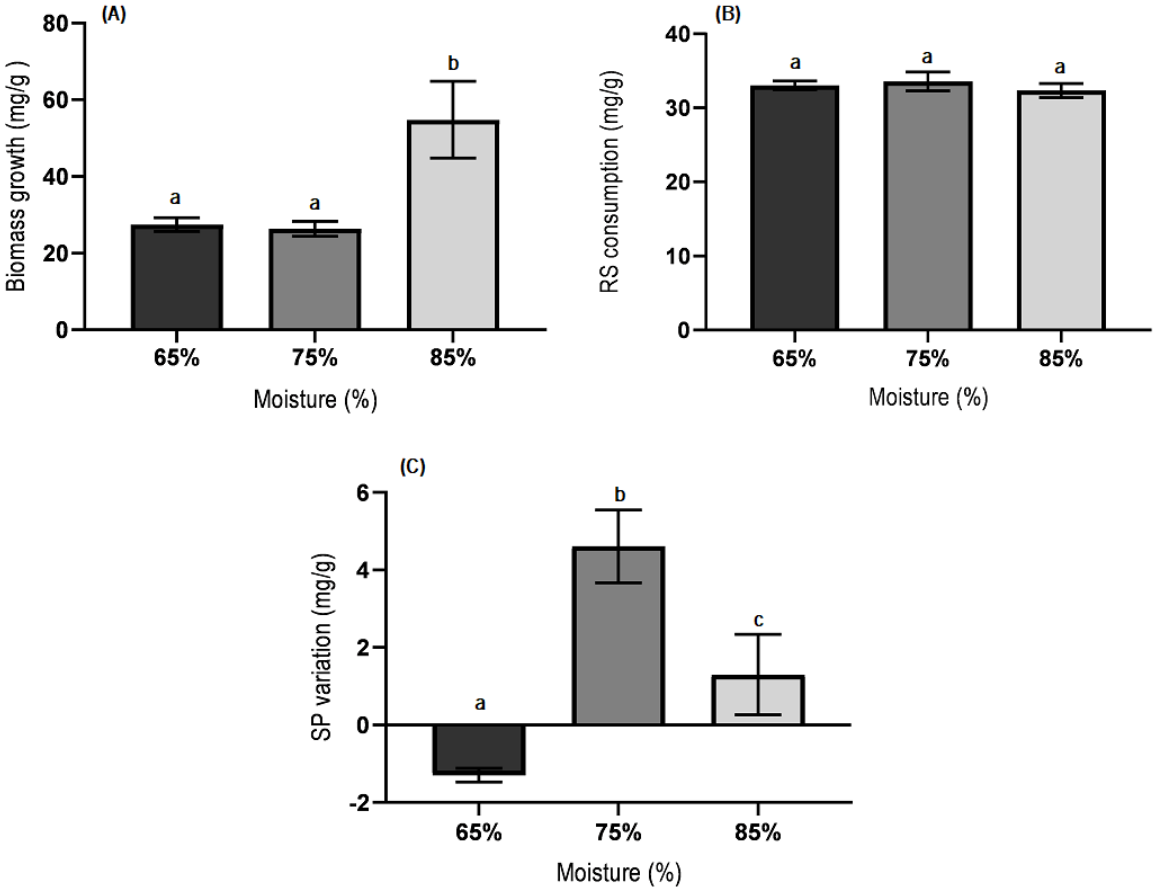

Figure 10. Effect of SSF with *C. cylindracea* using RSC+OP (2:1) as substrate on biomass growth (A), consumption of reducing sugars (B) and variation of soluble protein (C) (mg/g) over 4 days of fermentation using 3 distinct levels of moisture (65%,75% and 85%). In order to achieve values that represented the variation over the 4 days, in all concentrations was subtracted the concentration already present in the beginning of the SSF process. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

According to these results, the adjustment of the moisture content to 75% can be considered, the best condition for enzyme production (**Fig. 9**) after 4 days of SSF with the mixture of 66% RSC and 33% OP. Moreover, an acceptable growth (**Fig. 10 A**) and the greatest increase in soluble protein (**Fig. 10 C**), an especially interesting parameter to take into account if considering the increasing of nutritional value of the substrate, were also observed under this condition.

These results are dissimilar from others found in the literature using *Candida* species and agro-industrial residues as solid substrate. Rekha *et al.*, 2012 used groundnut oilcake as a substrate and obtained maximum activity of lipase for 60% moisture under fermentation with *C. rugosa* [97].

Similar values of 60% moisture were also obtained by Moftah *et al.,* 2012 when using *C. utilis* and olive oil cake for the optimal production of lipase and protease [35]. Other strains of microorganisms also revealed to have better production of these two enzymes under lower moisture levels [99], [100].

This difference between results may be associated with the different type of vegetable oil cakes or vegetable oil cakes mixtures used, or different strains reactions to the conditions used, as these optimal conditions for growth and enzyme production can vary from microorganism to microorganism.

Additionally, when comparing the enzymatic values obtained under these same conditions with these reported works, it can be noticed that they are relatively lower. However, contrary to what happened in this present study, most of the times in the available literature, the solid wastes used as substrates differ to others easier to valorize, due to its characterization and composition, furthermore, most of the time, they are also chemically enriched, which, improves the substrates nutritionally and consequently generates greater microorganism growth and better results [35], [97], [99], [100]. These chemical enrichments, however, represent a bigger cost for the valorization process.

4.1.5 Comparison between different substrate mixtures

In order to study and compare the reaction of the yeast to a different mixture (SFC+OP), SSF was carried out under the previous best condition only replacing the substrate used before by this new mixture in equivalent proportions (66% SFC and 33% OP (w/w)). Since RSC and SFC are very similar regarding their composition (**Table 4**) it was assumed that the ideal conditions for yeast growth and enzyme production would be also similar (time and ratios). However, two different moisture content percentages were tested - 75 and 65 % (w/w, wet basis) - since the latter also showed promising results in terms of lipase activity, and was in line with what was documented in previous works [35], [97], [101].

For lipase (**Fig.11 A**), there is a significant difference when comparing the two substrate mixtures at 65% moisture, being almost four times higher when using the new mixture with SFC ($P < 0.05$). When the moisture content was established at 75%, it is not noticed significant differences between the distinct mixtures ($P < 0.05$).

Furthermore, the 65% moisture condition with the mixture of SFC+OP proves to be the one where there is greater activity of the lipolytic enzyme and consequently greater production. These results are, more similar to the previously obtained and bibliographed for other yeasts of the same family that associate higher lipase activity values to lower percentages of moisture [35], [97], [101].

For proteolytic enzymes (**Fig.11 B**) and using this new substrate maximum activity values of 27.2 ± 0.6 in circumstances of 65% moisture, and 35.7 ± 2.6 U/g under 75% moisture conditions were reached, values significantly higher than those obtained using the substrate with RSC, if compared in the same conditions ($P < 0.05$). Additionally, it is noticed that protease production is favored under higher moisture conditions in both substrate mixtures.

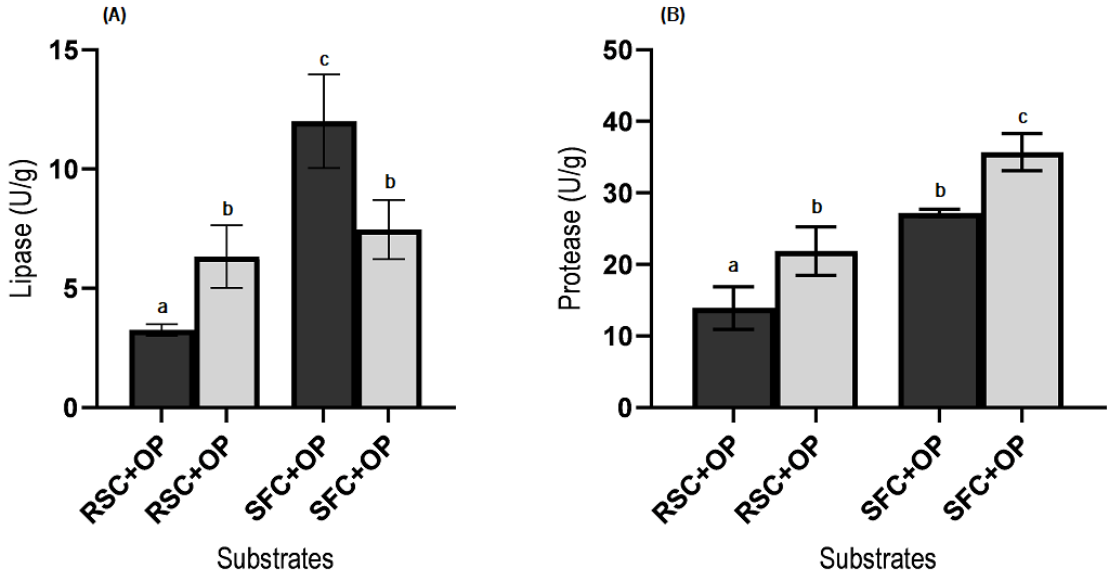

Figure 11. Lipase (A) and protease (B) activity (U/g) on SSF with *C. cylindracea* and an incubation time of 4 days using 2 levels of moisture - 65% (dark bars) and 75% (light bars) and 2 different substrates, RSC+OP (2:1) and SFC+OP (2:1). Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

After detailed visualization, it is possible to prove, regarding the yeast growth (**Fig. 12 A**), a significantly greater variation in biomass using the SFC+OP substrate with 75% moisture when compared with the others substrate mixtures, but also, even when compared to the same substrate in lower moisture percentages ($P < 0.05$).

This increased yeast growth using the SFC+OP, may be one of the reasons that help to explain the higher enzymatic activity (Observed in **Fig. 11**) obtained under this new substrate mixture when compared to the first used.

The consumption of RS, evaluated by the difference between the concentration of RS at the end of fermentation and at the beginning of SSF (**Fig. 12 B**) also proved to have significant differences ($P < 0.05$), with less sugars being consumed when the *C. cylindracea* uses the substrate of SFC+OP. Consequently, the yeast proves to have a more efficient growth when using this new mixture as substrate, since it growths the most and consumes less sugar, comparatively.

Concerning variation of SP (**Fig. 12 C**), no significant differences are worth to be noticed between the two substrate mixtures with 75% moisture. At lower moisture levels, there are, on the contrary, notable differences ($P < 0.05$), as the soluble protein variation is positive for the SFC+OP substrate mixture and

negative for the RSC+OP mixture, indicating once again a possible consumption by the yeast of the available protein in the medium in order to achieve cell growth.

In summary, the presence of SFC in the substrate mixture lead to improved yeast cell growth (**Fig. 12 A**) and resulted in greater enzyme activities (**Fig. 11**).

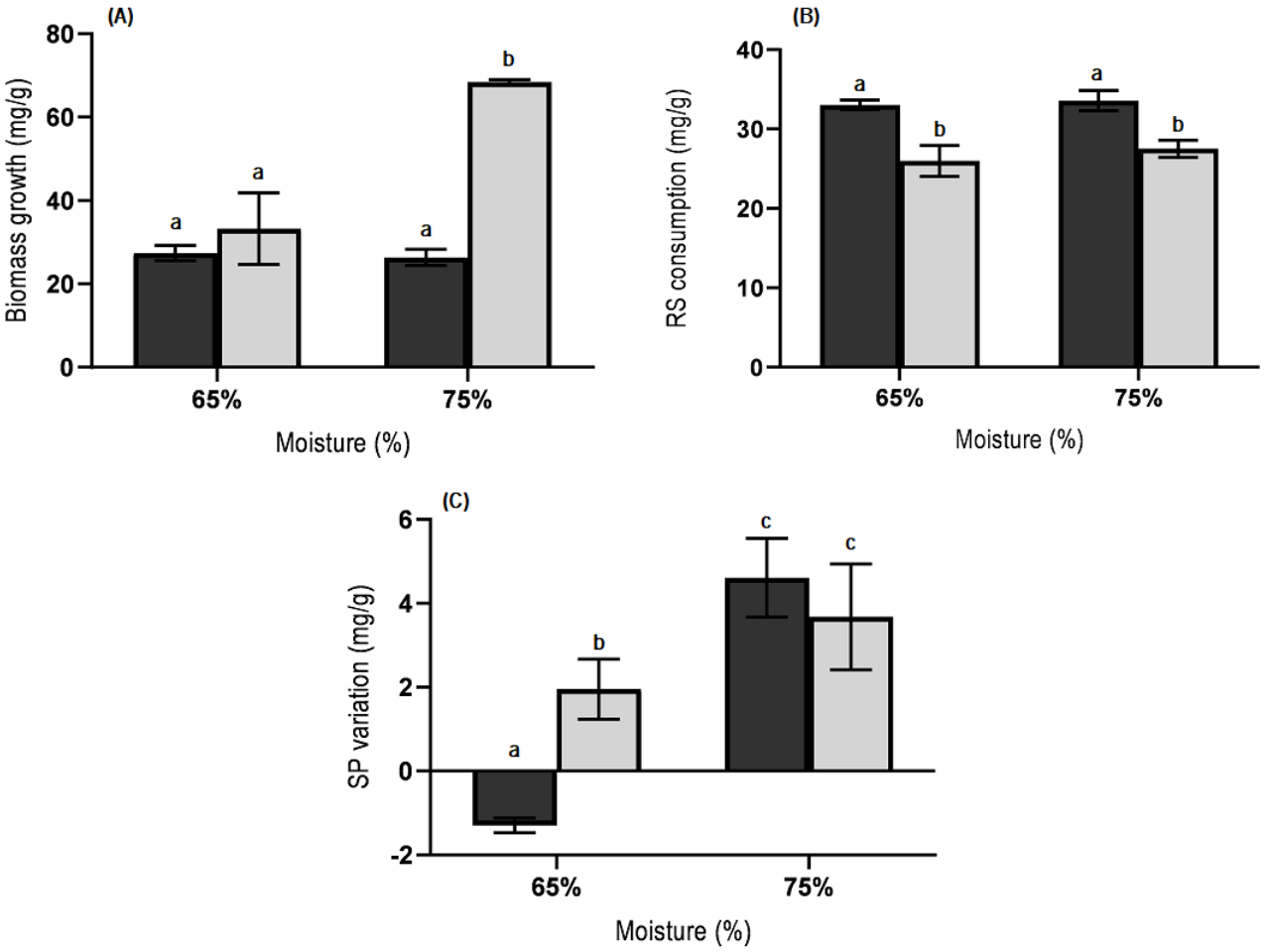

Figure 12. Effect of SSF with *C. cylindracea* using RSC+OP 2:1 (dark bars) and SFC+OP 2:1 (light bars) with two levels of initial moisture - 65% and 75% - on biomass growth (A), reducing sugars consumption (B) and soluble protein variation (C) (mg/g) over 4 days of fermentation. In order to achieve values that represented the variation over the 4 days, in all concentrations was subtracted the concentration already present in the beginning of the SSF process. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

4.2 *Trichoderma harzianum*

In SSF works, the objectives and final products that are often obtained also differ, depending on the type of microorganism used, which turns out to be the "engine" of the fermentation. Fungus are considered the best fitted microorganism to be used in SSF, as these fermentations simulate the conditions where they would normally grow, allowing them to synthetize several food and industrial enzymes (cellulases, xylanase, β-glucosidase, protease) that have a significant role in the nutrient valorization [102].

Therefore, SSF with a new microorganism was also studied, *T. harzianum* was the filamentous fungus selected to be optimized, as it is described in the literature as secretory of a wide variety of enzymes.

This time, a different methodological approach was used in order to study the use of this microorganism in the SSF, an experimental design: Box Benchen experimental design (BBD) with 3 levels (-1,0 and 1) and 3 factors (% moisture (w/w, wet basis), % RSC (w/w) and % SFC (w/w)). The experimental design allows for the direct optimization of the conditions, simultaneously (moisture and substrate proportions) in order to obtain the maximum responses values, in this case, enzymatic activity.

Also based on previous works and available information, where this same fungus or others identical, were used in SSF and SmF for the production of value added products or substrate nutritional enrichment, incubation time and temperature were set at 7 days and 28°C [60], [103], [104].

Using the BBD, 15 runs were performed for different responses of the following enzymes: lipase, protease, xylanase, cellulase, β-glucosidase and phytase. The responses are available in **table 5.** It was also observed that the best-fitted model for all enzymes was a quadratic model. With the responses obtained in the different runs (**Table 5**) and through the statistical program "Statgraphics 18" it is achieved different regressions equations for all enzymes optimizations (**Appendix 1**).

Table 5. Responses of the BBD experimental design (3 levels and 3 factors) of SSF using the filamentous fungi T. harzianum.

	Levels			Responses (U/g)					
Run	%Moisture	%RSC	%SFC	Lipase	Protease	Cellulase	Xylanase	β-glucosidase	Phytase
1	0	0	0	2.1	66.2	6.6	16.1	2.0	21.8
2	-1	-1	0	3.4	68.7	2.5	45.6	2.0	14.4
3	1	-1	0	0.9	85.1	1.3	19.5	0.4	42.9
4	-1	1	0	11.8	96.6	8.7	14.4	4.0	22.8
5	1	1	0	4.6	104.3	12.7	32.3	3.7	64.9
6	-1	0	-1	10.8	70.5	3.9	32.7	2.0	15.0
7	1	0	-1	1.3	41.3	0.9	18.7	0.2	13.8
8	0	0	0	6.9	67.6	5.1	15.1	0.5	30.2
9	-1	0	1	1.2	60.3	4.1	12.0	5.4	30.5
10	1	0	1	5.8	66.8	3.3	26.1	2.0	57.2
11	0	-1	-1	0	25.1	0	0	0.6	13.6
12	0	1	-1	3.8	90.3	8.3	18.1	2.4	22.7
13	0	-1	1	3.7	66.6	3.7	8.9	3.5	37.8
14	0	1	1	1.4	59.8	0.1	10.7	6.4	42.2
15	0	0	0	3.6	76.9	5.1	11.9	2.9	26.4

Level/factor	Moisture	%RSC	%SFC
-1	65%	0%	0%
0	75%	25%	25%
1	85%	50%	50%

4.2.1 Lipase and protease production

Through the experimental BBD, the response obtained at different levels and factors used, during the different fermentations was measured in their activity in the final extract. Considering the regression equations for lipase (**Lipase = 4.1 - 1.7*Moisture + 2.3*RSC - 0.7*SFC + 2.2*Moisture^2 - 1.1*Moisture*RSC + 3.5*Moisture*SFC - 1.4*RSC^2 - 0.6*RSC*SFC - 1.1*SFC^2**) and protease (**Protease = 70.2 + 1.7*Moisture + 15.4*RSC + 6.8*SFC + 2.3*Moisture^2 + 0.11*Moisture*RSC + 9.7*Moisture*SFC + 13.9*RSC^2 - 13.6*RSC*SFC - 13.6*SFC^2**), it is possible to see which factors had a positive and negative influence in the enzyme's activity. The same can also be confirmed in the pareto chart (**Fig. 13**) and 3D response surface (**Fig. 14**) presented next.

For lipase (**Fig.13 A**), only the combination of factors A and C - moisture content and SFC - reveal to be significantly relevant for its activity, influencing this response in a positive way.

In the case of protease (**Fig.13 B**), the isolated factor B - %RSC - reveals the ability to influence protease activity significantly in a positive way, while the combination of factors (CC and BC) influence this value in a negative way: the higher their value, the lower the response

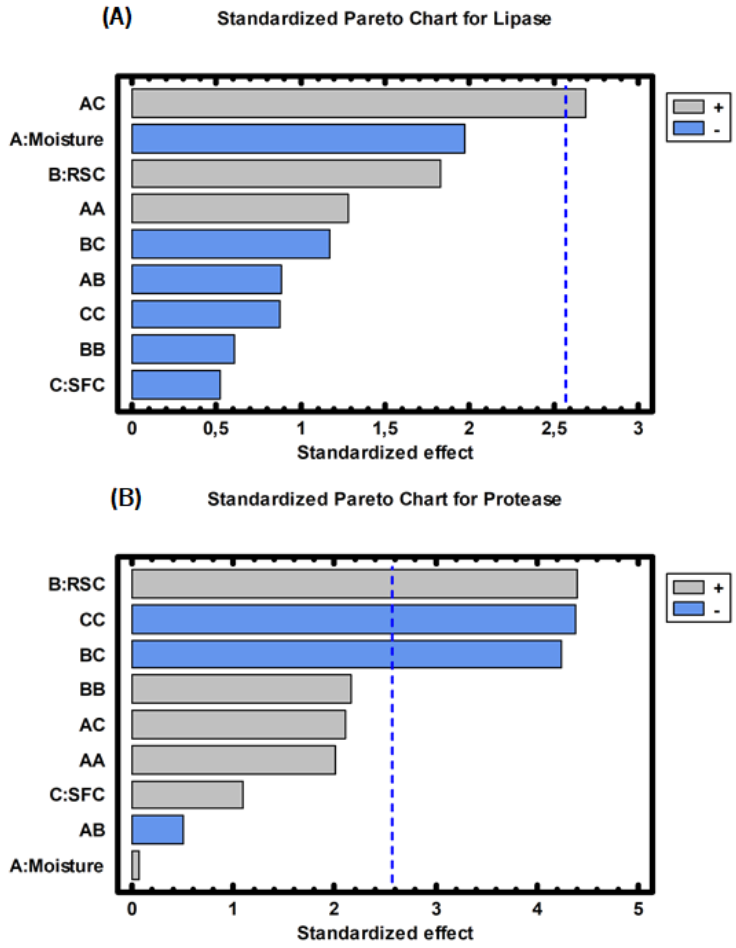

Figure 13. Pareto's chart representing the influence that each factor has on the response of lipase (A) and protease (B).

The 3D response surface graphics, in its turn, allow to easily visualize and help to confirm which factors have the higher influence in the final response and also what kind of influence they end up having in this response, negative or positive.

Fixing a factor to its optimum (C - %SFC) and alternating the others, it is possible to see that in the case of lipase (**Fig.14 A**), moisture content has a bigger influence than the percentage of RSC (B), since the curve presented regarding the first factor is more accentuated.

Concerning protease (**Fig.14 B**), the opposite effect is observed, with the factor B - %RSC - being the one that visually seems to have the greatest influence on the response surface and the moisture percentage (A) practically not influencing the final response, easily interpreted by the almost straight curve on the corresponding axis.

In the same figure (**Fig. 14**) is also possible to have the information about the approximate conditions that allow to obtain the greater activity of the respective enzymes (highlighted in both figures).

These conditions, where are obtained the maximum response values, are possible to be seen, on both cases, on the left, superior and farthest vertex (65% moisture and 50% of RSC in the substrate mixture).

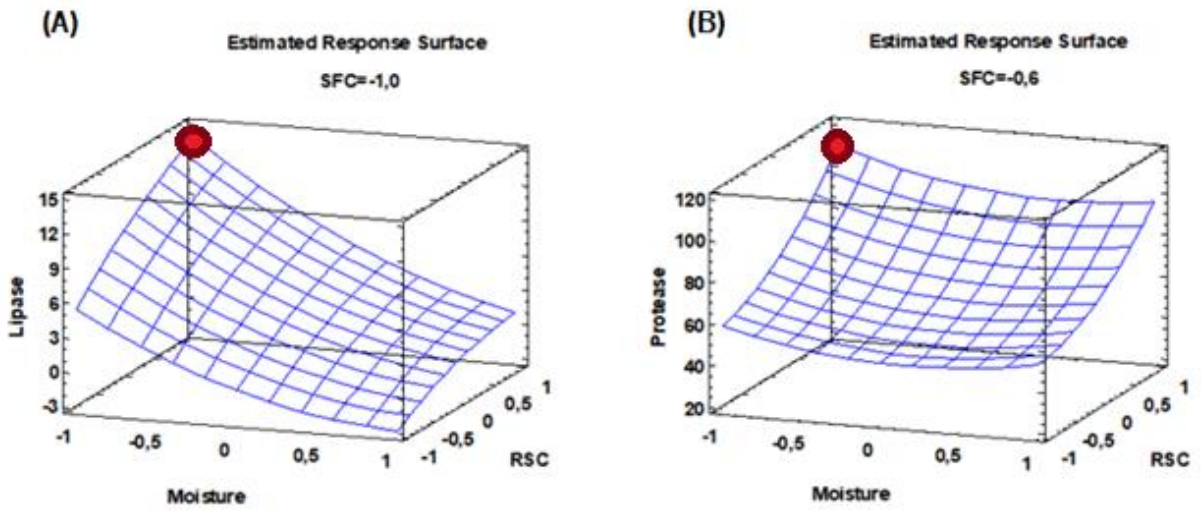

Figure 14. 3D surface plot of the interactions of the multiple factors (%moisture, %RSC and %SFC) on the response of lipase (A) and protease (B).

The final optimized conditions were determined by evaluating the effect of the three factors in study: moisture content and the percentage of RSC and SFC. Likewise, a substrate mixture using equal proportions of RSC and OP (50%) and a moisture content adjusted to 65 % appear to be the optimal conditions for lipase production under SSF with *T. harzianum.*

In turn, for optimal protease production, the moisture level is kept at 65% but the substrate composition is changed to a mix of 50% RSC, approximately 12.5% of SFC and the remaining percentage of OP (approximately 37.5%).

Furthermore, the model predicted that under the optimized fermentation conditions the maximum enzymatic activity for lipase and protease is 14 U/g and 108 U/g, respectively.

Concerning lipase, Toscano *et al.,* 2013 also reported the production of this enzyme using SSF and *T. harzianum,* obtaining maximum activities of 71.3 ± 1.5 U/g using an enriched medium with olive oil and wheat brand as a solid substrate [60]. The use of *T. harzianum* for lipase production under SSF was also tested by Coradi *et al.,* 2013 using a variety of agroindustrial wastes as substrate of castor bean cake, sugarcane bagasse, a mix of castor bean cake and cassava, a mix of castor bean and corn husk and a mix of castor bean and sugarcane. The maximum activity value reported by the authors was 4.0 U/g [66].

Not having many other reports of lipase production under SSF conditions using this fungus, the optimal value of lipase obtained using these substrates (14.2 U/g) can be considered a good and promising result, especially considering that the solid substrate used in this work did not suffer any kind of chemical enrichment, contrary to the one that obtained better results for the optimal production of this enzyme.

Comparing with the values obtained in the SSF with the yeast *Candida cylindracea*, the maximum lipase activity value with the fungus is roughly equal to the best activity value obtained using the yeast. Proving that for these two microorganisms there is not a big difference between their two maximum lipase activity values in optimal conditions using these by-products as substrate.

On the other hand, for the protease, the enzymatic activity value obtained using the *T. harzianum* when compared with the values obtained using the *C. cylindracea* in optimal conditions are very superior. Therefore, proving that under the tested circumstances, the fungus is a better producer of proteolytic enzymes, as described in the literature [4], [41], [48]

However, when compared with other reported works that also used filamentous fungi, like García-Gómez *et al.,* 2009 that used *Aspergillus niger,* or Vishwanatha *et al*., 2010 and Chutmanop *et al*., 2008, that used *Aspergillus oryzae,* the 108.3 U/g obtained in this assay using *T. harzianum* is comparatively lower [105]–[107]. This difference in the results was expected and justified by the use of a mixture with OP in their composition, an agroindustrial by-product not particularly rich for microorganism growth and enzyme production. Besides that, many times in these works where enzymes are produced, many other factors are optimized in addition to the moisture and mixture of the substrates.

4.2.2 Production of other enzymes (cellulase, xylanase, β-glucosidase and phytase)

In addition to the enzymes previously studied, filamentous fungi like the *T. harzianum* have the ability to produce another type of enzymes with high commercial interest, for example: lignocellulosic enzymes, namely cellulase, xylanase and β-glucosidase, or phytase, because of their excellent ability for extracellular protein production and capacity to readapt and survive in conditions where these enzymes are necessary [108]. It is currently estimated that approximately half of the enzymes used commercially and, in the market, originate from this type of microorganisms. [109]–[111]

Thus, the optimal production of cellulase, xylanase, β-glucosidase and phytase was also studied. Their responses to the different levels are presented in the **table 5**, and the respective regression equation obtained for each response presented in the **appendix 1**. Additionally, the response surface and respective interactions to the different levels and factors are presented in the pareto chart (**Fig. 15 and 16**) and in the 3D surface plot (**Fig. 17**).

For cellulase growth (**Fig. 15 A**), only B - % of RSC - has a significant and positive influence. Whereas for xylanase (**Fig. 15 B**), the combination of factors A and B - %moisture and %RSC - are those that influence its final activity, in a meaningful way. For the other lignocellulolytic enzyme (**Fig. 15 C**), the three individual factors addressed in the experimental design significantly influence its activity, however, contrary to all others, A - %moisture - influences this growth in a negative way.

Concerning phytase (**Fig. 16**), the individual factors C and A - %SFC and %moisture, respectively - are those that significantly influence enzyme production, with higher values of these two factors favoring its response (positive influence).

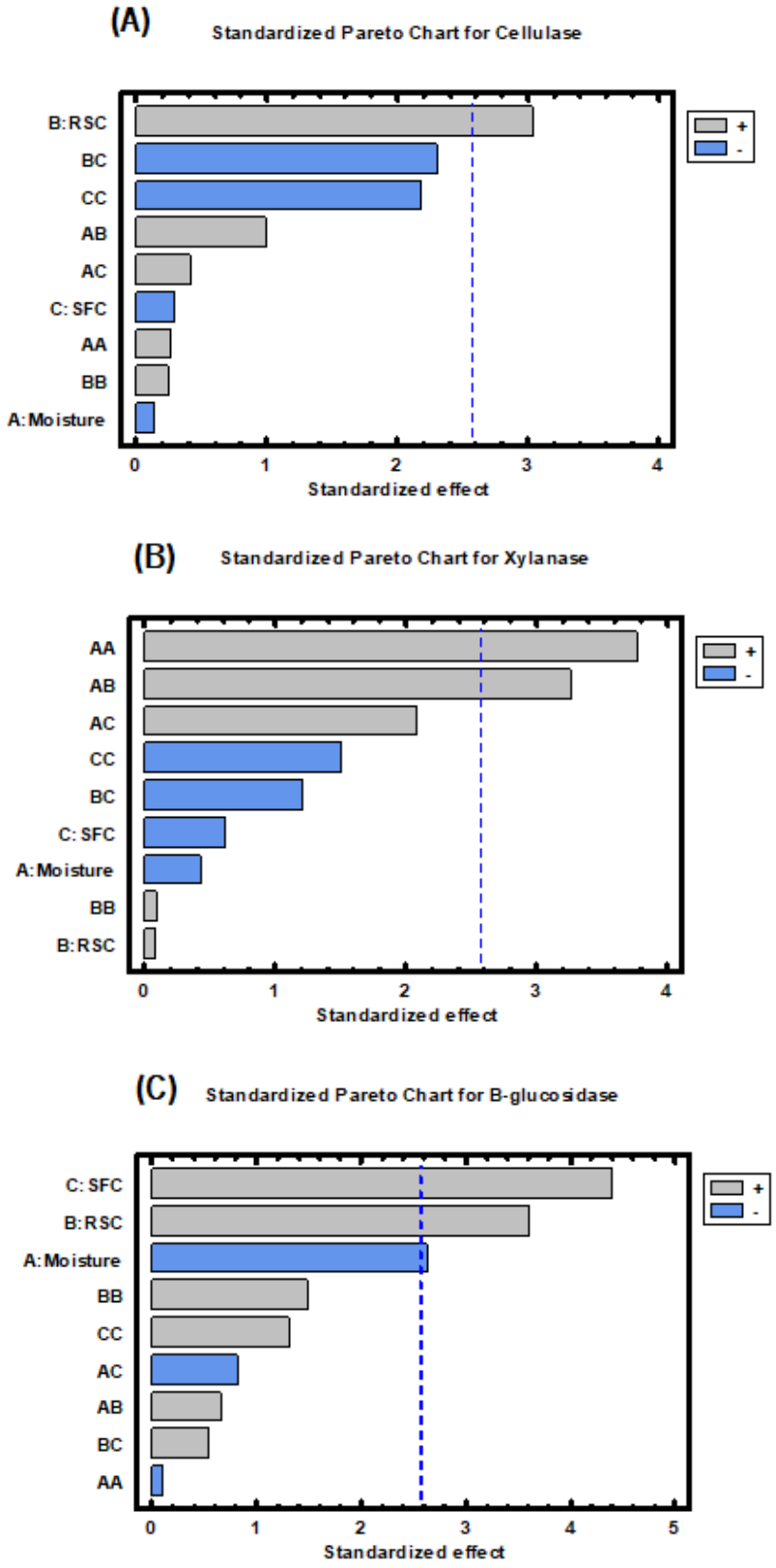

Figure 15. Pareto's chart representing the influence that each factor has on the activity of cellulase (A), xylanase (B) and β-glucosidase (C).

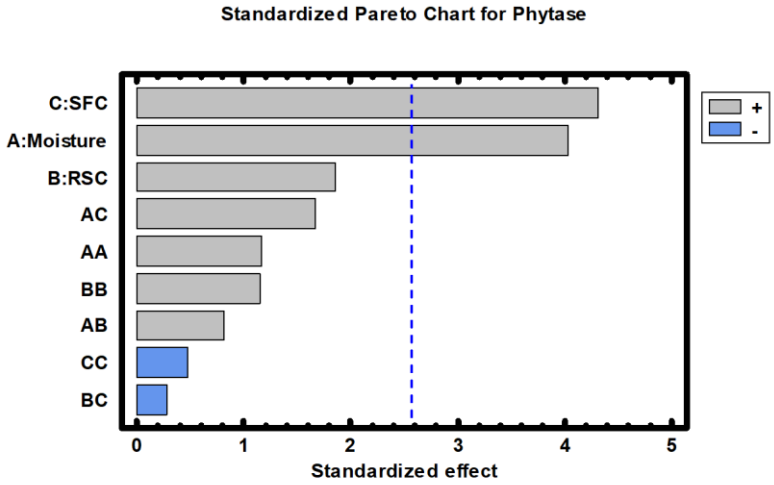

Figure 16. Pareto chart representing the influence that each factor has on the activity of phytase

The 3D plots presented in **figure 17** help to understand the interaction between the different factors and the response. Analyzing these plots, it is possible to observe that the conditions to produce the enzymes at optimal levels differ between each other. The optimal conditions for each enzyme are highlighted in their respective plot.

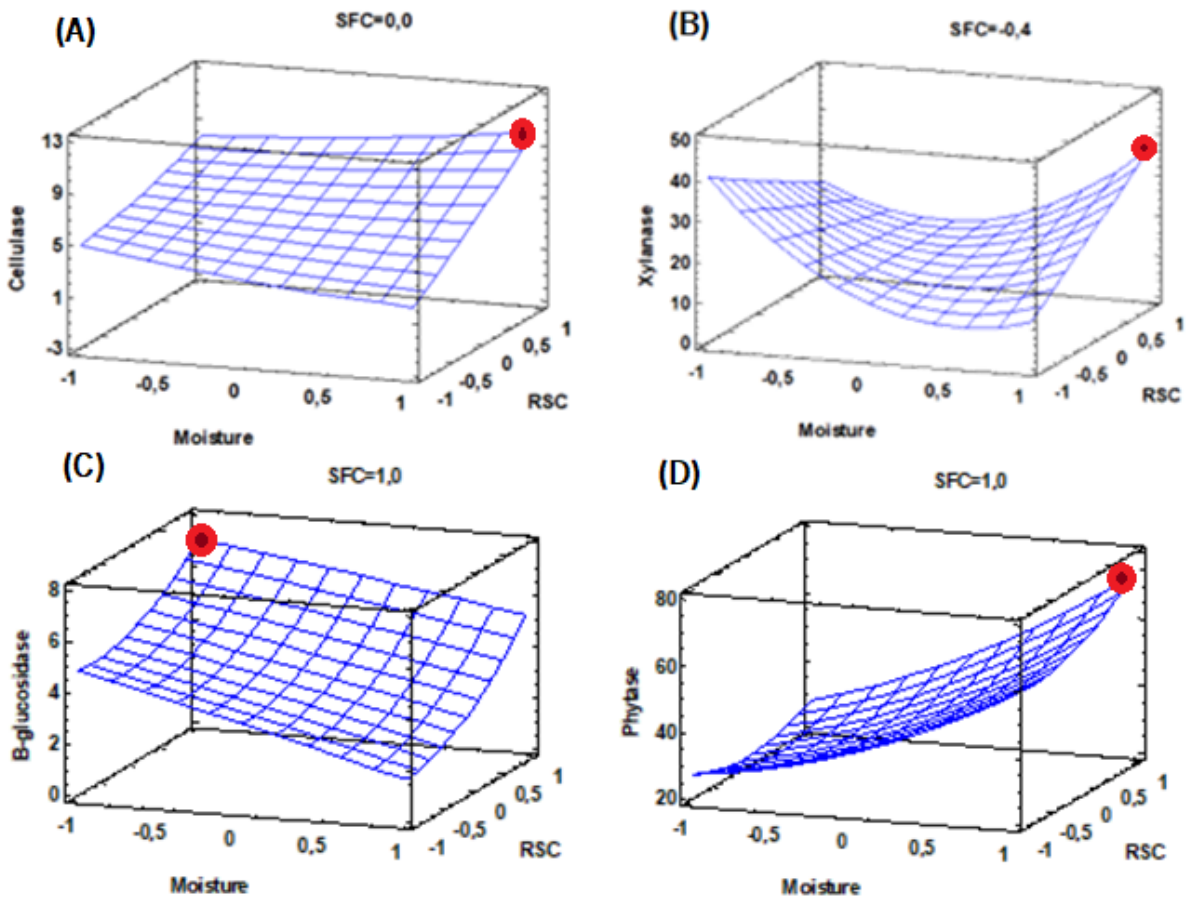

Figure 17. 3D surface plot of the interactions of the multiple factors (%moisture, %RSC and %SFC) on de production of cellulase (A), xylanase (B), β-glucosidase (C) and phytase (C) activity (U/g).

Using the BBD, the responses were optimized, and the optimal conditions that would translate in maximum enzyme activity for cellulase, xylanase, β-glucosidase and phytase, respectively, are presented next in **table 6**.

Table 6. Optimum levels, factors and activities for cellulase, xylanase, β-glucosidase and phytase

Enzyme	Optimum level			Optimum factor				Activity (U/g)
	%Moisture	%RSC	%SFC	%Moisture	%RSC	%SFC	%OP	
Celullase	1	1	0	85	50	25	25	10.9
Xylanase	1	1	≅ -0.5	85	50	≅12.5	≅37.5	40.7
β-glucosidase	-1	1	1	65	50	50	0	7.2
Phytase	1	1	1	85	50	50	0	73.7

In addition to these individual optimizations, the simultaneously optimization of lignocellulolytic enzymes: cellulase, xylanase and β-glucosidase, was also studied. The simultaneous production of these enzymes can increase the possible applications in industry, making them more commercially appealing and be a time/recourse saving factor.

The factors that allow their maximum simultaneously activities were predicted by the model, using BBD, and found out to be: (A) %moisture =1, (B) %RSC =1 and (C) %SFC ≅0,5. Which, in turn, ends up translating into real conditions of 85% moisture, and a substrate mixture compound with 50% RSC, 37.5% SFC and 12.5% OP.

In these conditions, the optimal values for cellulase, xylanase and β-glucosidase are 8.6 U/g, 37.8 U/g and 3.9 U/g, respectively. These Values are similar to those obtained for the individual optimization shown in **table 6.**

Rahnama *et al.* 2016 also used the *T. harzianum* for the production of lignocellulolytic enzymes with the agroindustrial waste of rice straw under SSF conditions and reported maximum values of 111.3 U/g, 433.8 U/g and 173.2 U/g for cellulase, xylanase and β-glucosidase, respectively [67]. Comparing the activities, the ones obtained in this work are lower. This difference is expected and explained by the use of OP as a component of the substrate mixture, a difficult by-product to be used by the microorganism as substrate.

In addition, the main objective of the work is not centered on the optimal and maximum production of enzymes, but on the enhancement of the OP mixtured with other cakes already used in animal feed in order to not only give OP a second life, but also, to allow a decrease in use of RSC and SFC in the animal diet. Therefore, by-products with more lignocellulosic content were not used (as the

rice straw, for example) which would allow higher values of enzymatic activity and more similar to those reported.

For the last enzyme considered, phytase, some other works on its production through SSF using not only *Trichoderma* species but also some others fungi and diverse substrates with agroindustrial origin have been published, for example Shivanna *et al.*, 2014 and Prado *et al.*, 2019 [88], [112]. The better results available in the literature are approximately identical to those obtained in this work (73.7 U/g), thus proving that these conditions used (substrates, moisture and microorganism) are promising for future works of phytase production.

5.Conclusions and future challenges

Currently, one of the biggest challenges facing humanity is the problem of excess waste as one of the main consequences of the rapid and uncontrolled phenomenon of world population increase. With this subject very present, the scientific community seeks to find workable solutions to give new uses to these residues, mainly those of agro-industrial origin, since they have already shown the suitability and characteristics to be reused in a viable way and, like this, alleviate some of the pressure that is felt on Earth and its natural resources.

SSF, a low-cost green biotechnological process is a perfectly reachable technology that uses these solid agro-industrial wastes as a substrate in an operation that over the last years has received increased attention due to their promising results regarding value added compounds production and treatment of these by-products.

Olive pomace, due to its composition, presents several challenges for biotechnological valorization, mainly due to the lack of nitrogen. In this work, the valorization of this agroindustrial by-product was tested with two different microorganisms along with two other substrates (rapeseed and sunflower cakes).

With regard to the results, for the yeast used, *Candida cylindracea,* the maximum production of lipolytic and proteolytic enzymes was verified under conditions of a substrate mixture of 66% (w/w) SFC and 33% (w/w) OP, with a fermentation period of 4 days and moisture conditions of 65% (w/w, wet basis) for lipase production and 75% for protease. The maximum activities values obtain for these two enzymes were 12 ± 2 U/g and 35.7 ± 2.6 U/g of solid substrate, respectively.

For the fungus *Trichoderma harzianum*, the experimental design Box-Benchen was used in order to select the optimal conditions simultaneously (substrate mixture and % of moisture) in order to produce lipase, protease, xylanase, cellulase, β-glucosidase and phytase. Under the optimal conditions for their respective production, the enzymatic activity values per gram of dry substrate obtained were: 14.2 U/g for lipase, 108.3 U/g for protease, 10.9 U/g for cellulase, 40.7 U/g for xylanase, 7.2 U/g for β-glucosidase and 73.7 U/g for phytase.

In general, the production of these enzymes demonstrated that the valorization of OP, mixed with the two other substrates (RSC and SFC) may enhance the value of OP and allow its integration in animal feed formulations replacing other ingredients, more expensive, of vegetable and animal origin. Moreover, the enzymes produced have important roles in improvement of feed digestibility.

In order to continue the work, it would be interesting to study how the SSF helps to increase the nutritional value of oil cakes, measuring the protein content, fiber carbohydrates among other factors in the final fermented and compare with the initial substrate. Additionally, it would be interesting to perform in vitro assays of protein and fiber digestibility, as well as to test these fermented OC in in-vivo trials in animal diets, in order to prove the possibility of OP incorporation in animal feed. To do in vivo trials higher amounts of fermented feedstuffs will be needed thus it would be interesting to perform scale-up experiments using different types of bioreactors, such as tray-type and rotary drum.

6. Bibliography

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7. Appendix

Appendix 1. Regression equations for the different responses to the BBD experimental design

Enzyme	Regression equation
Lipase	Lipase = 4.1 - 1.7*Moisture + 2.3*RSC - 0.7*SFC + 2.2*Moisture^2 - 1.1*Moisture*RSC + 3.5*Moisture*SFC - 1.4*RSC^2 - 0.6*RSC*SFC - 1.1*SFC^2
Protease	Protease = 70.2 + 1.7*Moisture + 15.4*RSC + 6.8*SFC + 2.3*Moisture^2 + 0.11*Moisture*RSC + 9.7*Moisture*SFC + 13.9*RSC^2 - 13.6*RSC*SFC - 13.6*SFC^2
Cellulase	Cellulase = 5.6 - 0.1*Moisture + 2.8*RSC - 0.3*SFC + 0.4*Moisture^2 + 1.3*Moisture*RSC + 0.5*Moisture*SFC + 0.3*RSC^2 - 2.9*RSC*SFC - 2.9*SFC^2
Xylanase	Xylanase = 14.4 - 1.0*Moisture + 0.2*RSC - 1.5*SFC + 13.3*Moisture^2 + 11.0*Moisture*RSC + 7.0*Moisture*SFC + 0.3*RSC^2 - 4.1*RSC*SFC - 5.3*SFC^2
β-glucosidase	β-glucosidase = 1.8 - 0.9*Moisture + 1.3*RSC + 1.5*SFC - 0.1*Moisture^2 + 0.3*Moisture*RSC - 0.4*Moisture*SFC + 0.8*RSC^2 + 0.3*RSC*SFC + 0.7*SFC^2
Phytase	Phytase = 26.1 + 12.0*Moisture + 5.5*RSC + 12.8*SFC + 5.1*Moisture^2 + 3.4*Moisture*RSC + 7.0*Moisture*SFC + 5.0*RSC^2 - 1.2*RSC*SFC - 2.1*SFC^2