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Additional Information

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

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Abstract

Vegetable oils are yearly produced in large amounts generating solid by-products after oil extraction, the oilseed cakes. These by-products are lignocellulosic materials that have been applied in animal feed with some limitations due to their high fiber content from the plant cell walls. Biotechnological processes can help overcome these limitations and contribute to up-grading or valorizing such by-products, enhancing their nutritional value as feed ingredients. In the present work, three oilseed cakes (sunflower, rapeseed and soybean) were used as substrate for solid-state fermentation (SSF) by filamentous fungi. A screening of fungi (*Aspergillus niger*, *Aspergillus ibericus* and *Rhyzopus oryzae*) for enzymes production and antioxidant compound liberation was performed. In all three substrates, *Aspergillus niger* MUM 2915 led to the highest production of cellulase per mass of dry solid (109 $U \cdot g^{-1}$), xylanase (692 $U \cdot g^{-1}$) and protease (157 $U \cdot g^{-1}$) and to the recovery of an extract rich in antioxidants, with the highest scavenging potential of free radicals and superoxide anion, chelation ability and reducing power. *Rhyzopus oryzae* produced the highest amount of β-glucosidase with a maximum of activity of 503 $U \cdot g^{-1}$ leading to an extract with the highest concentration of total phenolic compounds. All fungal species used in this work were able to decrease neutral detergent fiber (NDF) and acid detergent fiber (ADF) in all by-products. Through the principal components analysis, it was concluded that extracts with higher antioxidant potential were obtained in SSF with high enzymatic

activity. A positive correlation was established between the action of β-glucosidase and total phenolic concentration. These results, prove that SSF can improve the nutritional value of oilseed cakes by SSF through a decrease in cellulose and hemicellulose fractions of the cell wall of plant ingredients, while obtaining simultaneously added-value products, such as enzymes and antioxidant compounds.

Keywords: sunflower cake, rapeseed cake, soybean cake, lignocellulolytic enzymes, phenolic compounds, antioxidants, solid-state fermentation filamentous fungi.

Introduction

According to the latest report of the United Nations regarding population prospect, it is expected a global population of 9.73 billion people by 2050 and 11.2 billion people by the end of the century [1].

Alongside population growth there is an associated increase for food demand, thus overall food production must increase. Currently, Human diet is generally based in foods with high energy, fats, added sugars or salt with and inadequate/insufficient intake of fruits, vegetables and cereals [1]. This type of diet can result in high environmental impact due to the intense use of natural resources and may have negative effects on human health [2]. In this context, an improvement of production chains is mandatory. The reuse of agro-industrial wastes in animal feed can contribute to develop circular economy processes, with focus on recycling nutrients and increasing their nutritional value.

Vegetable oils are an important part of the Human diet as they constitute one of the main sources of energy. Hence, they have an unquestionable role in our daily lives due to their versatility and multiple applications in areas such as the food industry, cosmetics, pharmaceutical and also in the biofuels production [3,4]. Soybean, rapeseed and sunflower are the three main sources of oilseed for vegetable oils production [5]. According to the latest forecast from the United States Department of Agriculture (USDA) for the 2020/2021 season, global production of soybean is expected to reach 362.8 million tons [6]. Global rapeseed production, the second largest produced oilseed in the world, is expected to reach values near 70.8 million tons. The same report suggests an increased production of sunflower, achieving production values near 56.7 million tons which is a new production record regarding this oilseed.

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

cellulose, hemicellulose, pectin and lignin [7]. Additionally, non-structural carbohydrates such as low-molecular-weight sugars, oligosaccharides and starch comprise a significant fraction of these by-products.

Oilseed cakes (OC) are traditionally used for animal feed or plant/soil compost. These two options are seen as economically valuable and profitable ways of disposing by-products without any additional cost and with minimum environmental impact. The abundance of protein, carbohydrates and minerals make these by-products suitable to be employed as animal feed. In the Mediterranean area, the protein supplementation of ruminants is mainly performed by imported soybean meal [8–10]. In Europe rapeseed products are used in cattle, poultry and pig feed formulations. However, OC contain relatively high amounts of indigestible fiber fraction and lignin [11]. These fractions may hinder nutrient digestibility in animals. Additionally, the rapidly expansion of livestock sector increased the pressure on natural resources. More than one third of arable land is used for animal feed production.

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

OC are lignocellulosic materials, suitable to be used as substrate in biotechnological process like solid-state fermentation (SSF). SSF is defined as a fermentation process that occurs in the absence or near absence of free water using a natural or inert substrate as solid support and ensuring enough moisture to support the growth and metabolism of microorganisms. This fermentation mimics conditions close to the natural *habitat* of many microorganisms of which filamentous fungi are the ones that better adapt to SSF [12,13].

Sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC) have high protein content (30 to 50 %) and total carbon ranges between 45 and 50 %. Lignocellulosic fractions of these by-products can act as inductors or inhibitors for the production of extracellular enzymes in SSF, depending on their concentration [14]. This complex matrix is recalcitrant to degradation due to the covalent bonds between hemicellulose and lignin established by hydroxycinnamic acids such as the ferulic acid [15]. Phenolic compounds can be found within the matrix of plant cells in different forms: free phenolics, soluble bound (conjugated) or insoluble phenolics. Soluble bound are normally esterified to soluble compounds with low molecular weight such as carbohydrates, proteins or lipids [16]. Insoluble phenolics are also known as non-extractable phenolics and remain in the matrix of the residues after extraction of soluble phenolics [17]. These compounds can be found within the cell wall matrix of vegetable cells and can be released by

enzymes [18]. Phenolic compounds have potential health benefits due to their antioxidant properties such as electrophilic scavenging, inhibitors of reactive oxygen species (ROS) and metal ions chelation [19,20].

These cakes are produced every year in large quantities and are ready to use by-products. Due to their nature, SFC, RSC and SBC have the adequate properties to be used as substrate in SSF for the production of bioactive compounds with potential antioxidant activity. Also, through SSF it is possible to improve the nutritional value of these by-products to be used for animal feed. Additionally, the use of filamentous fungi allows the production of extracellular enzymes with commercial interest in several industries including as supplement in animal feed [21].

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

Materials and methods

Agro-food by-products

Three OC from the vegetable oils production industry were used: SFC, RSC and SBC. All OC were provided from companies operating in Portugal. SFC was provided by Sorgal, S. A. during the 2019/20 season and RSC and SBC were provided by IBEROL - *Sociedade Ibérica de Oleaginosas*, SARL. OC were dried at 65 ºC and stored at room temperature.

Microorganisms

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

Characterization of unfermented and fermented solid substrates

OC were characterized regarding their physical-chemical properties. Initial and post fermentation characterization of OC was performed to monitor the changes due to the fermentative process. Moisture was calculated by drying the substrates in an oven at 105 ºC during 24 h until constant weight. Ashes were determined by high temperature treatment at 575 ºC for 2 h in a muffle. Nitrogen content was analyzed by the Kjeldahl method from which crude protein was estimated using a defined factor of 6.25. Carbon was analyzed following the

process described by Leite, et al. (2016) [22]. Total lipids were extracted from 0.4 g of sample using a mixture of chloroform: methanol (2:1; v/v) as extraction solvent, according to Folch method [23]. The mixture was incubated at room temperature, with shaking for 1 hour. After, lipids were filtered through a glass wool and evaporated to dryness. The lignocellulosic composition (cellulose, hemicellulose and lignin) of by-products was calculated through a quantitative acid hydrolysis as described by Leite, et al. (2016)[22]. To estimate soluble protein, free sugars, total phenols and enzymatic activity an extraction with water (ratio solid: liquid, 1:5 w/v) was performed at room temperature with mechanical agitation for 30 minutes. Following, the extract was filtered through a nylon net and the liquid fraction was centrifuged at 2264 *g* for 10 minutes at 4 ºC. The enzymatic extract was recovered and stored at -20 ºC until its analysis. Soluble protein was measured by the Bradford method and free sugars were analyzed by the 3,5-dinitrosalicylic acid (DNS) method. Total phenols were quantified using the Folin-Ciocalteau method (Commission Regulation (EEC) No. 2676/90).

Antioxidant activity

Antioxidant activity assessed by the DPPH method was performed as described by Dulf et al. (2015) with some modifications [24]. Briefly, 200 μL of different sample extracts were added to a 96 well microplate alongside with 100 μL of a methanolic solution of DPPH (0.5 mM). The mixture was left standing in the dark at room temperature for 30 min. Then, the decrease of absorbance of DPPH was read at 517 nm. Methanol was used as blank and DPPH solution without test samples served as control. Known amounts of Trolox were used to construct a calibration curve. The free radical scavenging activity of the extracts was expressed as micromoles of Trolox equivalents per gram of dry substrate ($\mu mol \cdot g^{-1}$).

The ICA method was performed as described by Oliveira et al. (2018) [25]. 50 μL of different concentrations of sample extracts were added to a solution of 0.12 mM of $FeSO_4$ (50 μL) and 50 μL of Ferrozine (0.60 mM). The mixtures were shaken and left at room temperature for 10 min. After 10 min, the absorbance of the solution was read at 562 nm. Blank sample was performed as described, without the addition of Ferrozine solution. A mixture of $FeSO_4$, Ferrozine and ultrapure water was used as control. Known amounts of EDTA were used for calibration. The ability of extracts to chelate ferrous ion was calculated as the % of reduction of absorbance compared to the control and expressed as EDTA equivalents ($nmol \cdot g^{-1}$).

The SOD was determined using the PMS-NADH non-enzymatic assay according to Gangwar et al. (2014) with some modifications [26]. Briefly, 50 μL of different concentrations of sample extracts were added to 50 μL of NADH (166 μM), 150 μl of NBT (43 μM) and 50 μL of PMS (2.7 μM), in a 96 well microplate, to a final volume of 300 μL. All components were dissolved in phosphate buffer (19 mM, pH 7.4). The mixture was shaken and left in the dark between 7

and 10 min. Then, the absorbance of the solution was read at 562 nm. Known concentrations of ascorbic acid were used for calibration. The scavenging activity of the extracts was expressed as micromoles of ascorbic acid equivalents per gram of dry substrate ($\mu mol \cdot g^{-1}$). FRAP was performed according to Benzie and Strain (1996) with some modifications to adapt the assay for a 96-well microplate [27]. The FRAP reagent was prepared by mixing 25 mL of 300 mM acetate buffer, 2.5 mL of 2, 4, 6 – tri (2 – pyridyl) – S- triazine (TPTZ) 10 mM solution and 2.5 mL of iron (III) chloride 20 mM solution in a ratio of 10:1:1. 20 μL of sample were mixed with 150 μL of FRAP reagent. Mixture was kept in the dark for 10 minutes. The complex formed by ferrous tripyridyltriazine (Fe^{III}-TPTZ) is reduced to the complex ferrous tripyridyltriazine (Fe^{II}-TPTZ) in the presence of antioxidants. This complex has an intense blue color with a maximum absorption at 593 nm. All sample analysis was performed in duplicate.

Hydration properties

Water holding capacity (WHC) was analyzed as according to Raghavendra et al. (2004) [28]. WHC was quantified by hydrating 1 g of dry sample with 30 mL of distilled water. The mixture was kept at room temperature for 18 h with stirring. The suspension was filtered, and the hydrated solid was dried at 105 ºC for 2 h to obtain the residual dry weight. Values were expressed as gram of water per gram of dry solid.

SSF of agro-food by-products

SFC, RSC and SBC were used as substrate in SSF experiments to evaluate the effect of three fungi species, *R. oryzae*, *A. ibericus* and *A. niger*. SSF was carried out in 500 mL Erlenmeyer flasks with 10 g of dry substrate. Moisture was adjusted to 75% (w/w, wet basis) with distilled water. Erlenmeyer flasks with the substrates were sterilized at 121 ºC for 15 minutes. For inoculation, the fungi grown on PDA for 7 days were suspended in a sterile solution (1 $g \cdot L^{-1}$ peptone and 0.1 $g \cdot L^{-1}$ Tween 80) and adjusted to a spore concentration of 10^6 $spores \cdot mL^{-1}$. Each substrate was inoculated with 2 mL spore suspension and incubated at 25 ºC for 7 days. A control experiment was performed at the same SSF conditions but without inoculation. Each experiment was performed in duplicate.

Enzymatic activities

Cellulases (endo-1,4-β-glucanase) was determined using carboxymethylcellulose (CMC) as substrate. 250 μL of 2% (w/v) cellulase substrate in 0.1 M sodium acetate buffer, pH 4.5 was mixed with the same volume of enzyme containing sample. After enzymatic hydrolysis, reducing sugars were quantified by the DNS method. One unit of enzyme activity was defined

as the amount of enzyme required to release 1 μmol of glucose reducing sugar equivalents from CMC in 1 min at 50 ºC and pH 4.5. The values of cellulases activity were expressed in units per gram of dry substrate ($U \cdot g^{-1}$).

The procedure to determine xylanases activity was the same as described for cellulases activity but the substrate solution used was xylan from beechwood 1% w/v and the reaction time was 15 min instead of 30 min. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of xylose reducing sugar equivalents from beechwood xylan in 1 min at 50 ºC and pH 4.5. The values of xylanase activity were expressed in units per gram of dry substrate ($U \cdot g^{-1}$).

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

Proteases activity was quantified using azo casein (0.5 % w/v) as substrate, in sodium acetate 50 mM, pH 5. 500 μL of sample was mixed with the same volume of substrate solution and incubated at 37 ºC for 40 minutes. Afterwards, 1 mL of trichloroacetic acid 10 % w/v was added to stop the reaction. Samples were centrifuged for 15 minutes at 600 *g*. Supernatant was recovered and after the addition of 1 mL of 5 N potassium hydroxide, the absorbance was read at 428 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of azopeptides in 1 minute at 37 ºC and pH 5. Values of protease activity was expressed in units per gram of dry substrate ($U \cdot g^{-1}$).

Statistical analysis

Principal component analysis (PCA) and statistically significant differences analysis were carried out using Statgraphics Plus 5.1(Manusgistics, Inc., Rockville, MD).

Results and discussion

Characterization of oilseed cakes

OC were physically and chemically characterized to assess their potential as substrate in SSF. **Table 1** shows the initial composition of OC.

OC used in this study are natural sources of protein, corresponding to 40–50 % of the total solid. Nitrogen (N) and carbon (C) content are important parameters to take into account when assessing if a substrate is suitable to be applied in SSF. N stimulates fungal conidiation, while C

represents the energy source to support microbial growth and metabolism [29]. However, it was proven that the ratio of both contents (C/N) have higher significant effect in fungal growth and sporulation than C sources alone. It is estimated that and optimal C/N ratio is between 10-20, depending on the fungus strain [30–32]. Sunflower cake (SFC), rapeseed cake (RSC) and soybean cake (SBC) are rich in N and presented a suitable C/N ratio to be used as substrate in SSF without the need for external N supplementation. RSC and SBC C/N ratio were within the average values reported in the literature [33–35]. SFC has a lower C/N ratio attributable to a higher N content. It is known that soil composition and fertility, location, rainfall or temperature can influence the productivity and composition of crops. OC obtained after mechanical press contain small amounts of residual oil or fat, referred to as lipids. The OC treatments after oil extraction have a significant impact in the final OC composition. The amount of lipids in the present OC accounted for approximately 2 % of dry weight. Srilatha and Krishnakumari (2003), reported a variation of 2.75-fold in fat content between dehulled and whole SFC. A smaller difference (1.41-fold) was observed between dehulled and partially dehulled SFC [36].

Cellulose and hemicellulose content were similar in the three OC while, SFC and RSC presented a higher concentration of lignin. Cellulose and hemicellulose fractions can act as inductors for the production of lignocellulolytic enzymes such as cellulases and xylanases, respectively [22]. These enzymes are responsible for the degradation of cellulose and hemicellulose respectively, increasing OC digestibility in livestock. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) are good indicators of feed quality once they are correlated with digestibility by animals. They are good indicators of dietary energy and intake, with focus for ruminant rations. However, the establishment of standard values for each fraction is not correct once fiber is not chemically, physically or nutritionally uniform material [37]. All three OC presented similar values of NDF and ADF and were within the range of the values reported by literature with the exception of SBC that present slightly higher values. All the OC retained water. This is a critical point in SSF. Water holding capacity (WHC) represents the water absorbed by the substrate under limiting conditions and that can be used by the fungi to support its growth [38,39]. Substrates used in SSF process usually have low water content. However, substrates with high WHC are normally selected for being used in SSF once the moisture can be easily modified according to the process needs. Ngoc et al. (2012) reported values of WHC for different fiber-rich plant sources and agro-industry by-products ranging from 2.5 and 8.0 ($kg \cdot kg^{-1}$ of dry matter) [40]. WHC is related with the content of lignocellulosic fractions. SBC showed a lower WHC compared to the reported in literature and this may be explained by the low concentrations of hemicellulose and cellulose. All cakes in this study had a similar pH value, around 5, which is found to be within the optimal range for filamentous fungal growth (3.8 to 6.0) [29]. The three OC contain small fractions of free extractable compounds such as phenolics, sugars and proteins. In the present work, these fractions were obtained through an

aqueous extraction. These compounds are easily available to fungi and can induce or inhibit initial fungal growth. In addition they can also act as repressors of enzyme synthesis [41,42].

Antioxidant potential of oilseed cakes

Phenolic compounds are amongst the secondary metabolites produced by plants [43]. **Figure 1A** represents the concentrations of total phenolic compounds (TPC), obtained after aqueous extraction, of each OC. SFC is the by-product with higher content of TPC. Its concentration is 1.94-fold higher than RSC and approximally 2.73-fold higher than SBC. It is known that OC contain high fractions of phenolic compounds. However, the presence of some of these compounds can hinder their use as animal feed as they can negatively affect protein digestibility from OC due to complex protein-phenolic compound interactions [44]. On the other hand, other phenolics may present relevant bioactivities, since they may provide health benefits due to antioxidant properties, induction or inhibition of enzymes and gene expression [45,46]. **Figures 1B-E** depicts the results of antioxidant analysis of sample extracts. Regarding capacity to scavenge the DPPH radical (**Fig. 1B**), the maximum value was obtained in SFC extract, that was 2 and 6-fold higher than the values obtained with RSC and SBC respectively. The antioxidant potential of each extract is in agreement with the concentration of TPC. Teh et al. (2014) suggested that the antioxidant capacity was proportional to the concentration of phenolic and flavonoid compounds in extracts of OC [47]. **Figure 1C** represent samples ability to chelate iron. Iron chelators may function as antioxidants by scavenging reactive oxygen species (ROS). Additionally, they can reduce the available iron resulting in a decrease of hydroxyl radical generation through Fenton reactions [48]. Aqueous extracts of SFC had the highest capacity to chelate iron, and the values obtained with SFC and SBC did not present statistically significant differences ($p < 0.05$). The low capacity to chelate iron of RSC extracts may be explained by a different nature of phenolics present in this extract. Iron is an element that can undergo cyclic oxidation and reduction and is involved in the Fenton reaction leading to the production of ROS that are capable of oxidizing a range of organic substrates [49]. ROS are formed as natural by-products of normal cell activity [50]. Superoxide anion is formed within the mitochondria, peroxisomes and endoplasmic reticulum [51–53]. This anion is one of the three primary species of ROS, alongside hydrogen peroxide and hydroxyl radical. They can react with organic substrates leading to the production of intermediate species able to produce more ROS [54]. From **Figure 1D** is possible to observe that again SFC extracts presented the highest superoxide anion scavenging capacity among the three OC extracts. SFC scavenging capacity was 4.8-fold higher than RSC and SBC, with no statistically significant differences ($p < 0.05$) between the results among these two OC. **Figure 1E** show the results of FRAP assay in OC extracts. This method is based on the antioxidant strength in reducing the ferrous tripyridyltriazine (Fe^{III}-TPTZ) complex

to its stable form (Fe^{II}-TPTZ). Extracts that exhibit antioxidant capacity in this assay, normally are electron donors as they reduce the Fe^{III}-TPTZ to its stable form interrupting the oxidation chain [55]. Results follow a similar trend as the ones obtained through the DPPH assay. Statistically significant differences ($p < 0.05$) were observed between the three extracts. SFC extracts have the highest reducing power, followed by RSC. A difference of 1.86-fold was observed between SFC and RSC. SBC extract showed the lowest reducing power being 11-fold lower than SFC.

From these OC extracts characterization, it may be concluded that SFC is the OC with more potential for antioxidant compounds extraction by an eco-friendly solvent (water).

Production of enzymes by SSF

OC were used as substrate in SSF with three fungi species. **Figure 2** show the activity of cellulases (**A**), xylanases (**B**), β-glucosidases (**C**) and proteases (**D**) obtained after SSF. Cellulases maximum activity, was achieved with *A. niger*, using SBC as substrate. *A. niger* was in general the best producer of cellulases in the three OC at the SSF conditions used, however no statistically significant differences ($p < 0.05$) were found between cellulases produced by *A. niger* and *A. ibericus* using SFC as substrate. Maximum xylanases activity was obtained using RSC as substrate fermented by *A. niger* and this enzyme production was 66 %, 85 % and 69 % (SFC, RSC and SBC, respectively) higher than the obtained with *A. ibericus*, that was the second largest producer.

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

The use of SFC and canola (a cultivar of rapeseed) for the production of cellulases and xylanases, using different *Aspergillus* species, was evaluated by de Castro et al. (2011) [34]. Maximum enzyme activity for these substrates was achieved using *A. awamori* IOC-3915. They reported activity values of 9.7 $U \cdot g^{-1}$ and 6.1 $U \cdot g^{-1}$ for cellulases and 413.5 $U \cdot g^{-1}$ and 571.4 $U \cdot g^{-1}$ for xylanases activity, respectively for each substrate. *A. niger* IOC-4003 produced cellulases activity of 1.1 $U \cdot g^{-1}$ and 0.2 $U \cdot g^{-1}$ and xylanases activity of 40.6 $U \cdot g^{-1}$ and 13.7 $U \cdot g^{-1}$ using SFC and canola cake as substrate, respectively. The use of soybean meal as substrate for a multienzyme production was evaluated by Vitcosque et al. (2012) [56]. They observed a maximum xylanases production of 47.7 $U \cdot g^{-1}$, using *A. niger*. Despite the production of β-glucosidases using OC as substrate for SSF has not been yet extensively explored, studies using other lignocellulosic materials to produce this enzyme have been performed. Leite et al. (2019), reported a maximum activity of 93.66 $U \cdot g^{-1}$ of β-glucosidases for *A. niger* CECT2088 using

brewer's spent grain as substrate [18]. Filipe et al. (2019), optimized the production of β-glucosidases using olive mills and winery wastes as SSF substrate, achieving maximum enzyme activity of 25.5 $U \cdot g^{-1}$ and 17.9 $U \cdot g^{-1}$ for *A. ibericus* MUM 03.49 and *A. niger* CECT 2915 [57], respectively. R. Ezeilo et al. (2019), reported that *R. oryzae* presented maximum β-glucosidases activity of 145.47 $U \cdot g^{-1}$ after optimization of SSF parameters, using raw oil palm frond leaves as substrate [58].

Apart from lignocellulolytic enzymes produced by filamentous fungi during SSF, several other enzymes can be produced using this process. Proteases are among the wide spectrum of enzymes that can be produced by SSF [59].

Maximum protease activity was achieved with SFC and *A. niger* however, showing no statistically significant differences ($p < 0.05$) with the ones obtained with the other fungi in SFC. SSF of RSC and SBC led to lower production of protease with all the fungi than the obtained with SFC but *A. niger* was the best producer ($p<0.05$) in these OC. *A. niger* is one of the most important sources of fungal proteases once, its genome sequencing by Pel et al. (2007) revealed 198 proteins involved in proteolytic degradation process [60]. Castro et al. (2011) reported proteases production of 0.8 and 0.5 $U \cdot g^{-1}$ using SFC and canola cake as substrates for SSF [34]. In the same work it was reported maximum proteases production of 9.9 and 12.6 $U \cdot g^{-1}$, using SFC and canola cake as substrate for SSF when fermented with *A. awamori* IOC-3915 and *A. sulphureus* respectively. Gupta et al. (2018) reported a maximum protease activity of 52.5 $U \cdot g^{-1}$, using *madhuca indica* (mahua) cake as substrate for SSF, using *A. niger* [61]. Mukhtar and Ikram-ul-Haq (2009) achieved maximum protease activity of 5.2 $U \cdot g^{-1}$, 3.3 $U \cdot g^{-1}$ and 4.8 $U \cdot g^{-1}$of dry substrate, for sunflower meal, rapeseed meal and soybean meal, respectively, using *A. niger* IGH_9 [62].

Enzymatic activity values achieved during this work easily outpace the ones reported by other authors. Cellulase maximum activity of 109 $U \cdot g^{-1}$ exceed the maximum production of 84.2 $U \cdot g^{-1}$ reported by Filipe et al. (2019), by 1.3-fold [57]. Regarding xylanase, an increased difference of 100 $U \cdot g^{-1}$ was obtained comparatively to the maximum of 598 $U \cdot g^{-1}$ reported by Castro et al. (2011) [34]. With concern to β-glucosidase production a difference of approximally 71% was observed between the maximum of 145 $U \cdot g^{-1}$reported by R. Ezeilo (2019) and the maximum obtained in this work of nearly 503 $U \cdot g^{-1}$ [58]. Additionally, protease maximum production of 157 $U \cdot g^{-1}$ was around 3-fold higher than the maximum of 52.5 $U \cdot g^{-1}$, reported by Gupta et al. (2018) [61].

Antioxidant capacity of extracts of fermented oil cakes

TPC and antioxidant potential of aqueous extracts of fermented OC were evaluated and compared with unfermented sterilized OC. According to the results in **Figure 3A,** SSF had a

positive effect, comparing to controls, on the increment of TPC concentration in the extracts obtained with all the fungi in RSC and SBC, and with *R. oryzae* in SFC, where the action of the other fungi did not affect the TPC liberation. For SSF with *R. oryzae* the increase of TPC in the extracts compared to controls was approximally of 2.6-fold in SFC, 5.6-fold in RSC and 10-fold in SBC. These results indicate the importance of SSF for TPC extraction of OC that are more resistant to direct extraction process with eco-friendly solvents.

TPC increase during SSF may be related with the action of lignocellulolytic enzymes. Insoluble phenolic compounds can be found connected to structures of the vegetable cell wall such as arabinose or galactose residues of hemicellulose components and also to lignin, which is a network of phenolic compounds [63–65]. Filamentous fungi possess two extracellular enzymatic systems, an hydrolytic one that produces hydrolases, able to degrade polysaccharides and an oxidative system, responsible for the degradation of lignin and opening of phenyl rings, increasing free phenolics [66]. The decomposition of linkages between lignin, cellulose or hemicellulose increase the release of phenolic compounds [67].

Highest concentration of TPC where observed in fermentation performed with *R. oryzae* which, also reported the maximum β-glucosidase activity. Sheih et al. (2014) observed that β-glucosidase, alongside α-amylase were responsible for the mobilization of phenolics during *A. niger* M46 fermentation [68]. Schmidt et al. (2014) suggested that the use of the fungus *R. oryzae* in rice bran could produce enzymes able to release phenolics such as ferulic acid [69].

Figures 3B-E show the results obtained of four different antioxidant assays performed with OC fermented extracts to assess their antioxidant potential. Regarding the ability to scavenge free radicals (**Fig. 3B**), maximum antioxidant potential was obtained in aqueous extracts of RSC fermented with *A. niger*, that was the fungi with which highest values of antioxidant potential were achieved in fermentations performed with all OC. *A. niger* was able to increase antioxidant potential of extracts in approximally 2, 4.6 and 14.3-fold for SFC, RSC and SBC, respectively. Surprisingly, *R. oryzae* fermented extracts achieved lower values of antioxidant potential than *A. niger*. These results indicate that the phenolic compounds that are liberated by the action of these fungi species are different and may be related with the action of enzymes most produced by each species, as it was found in this work. In fact, the direct correlation of TPC and antioxidant capacity may be difficult to establish as it found by Terpinc et al. (2012) that studied the correlation between TPC and antioxidant potential of different oil cake extracts [70]. They observed a negative correlation between these two parameters. Previously, Kähkönen et al. (1999), also reported that no significant correlation could be found between TPC and antioxidant activity of various plant extracts [71].

Additionally, Terpinc et al. (2012) suggested that samples with similar concentrations of TPC vary significantly in their antioxidant activity. The same was observed in this work between fermented extracts of *A. ibericus* and *A. niger*. This fact may be explained by the synergistic and

antagonistic interactions between the antioxidants. The action of lignocellulolytic enzymes such as cellulases and xylanases can also lead to antioxidant potential differences once the hydrolysis of some phenolic conjugates, present in the lignocellulosic matrix of plant cell walls, can release free phenolic compounds and low molecular weight molecules with higher antioxidant potential [72].

The highest antioxidant potential of extracts from fermented OC with *A. niger* was also observed in the results of the ability to chelate iron (**Fig. 3C**) were the maximum capacity was observed in fermented extracts of SBC. Generally, SSF increased capacity to chelate iron of the extracts compared to the control, with the exception of SFC when fermented with *A. ibericus*.

With respect to the superoxide scavenging potential (**Fig. 3D**), fermented extracts showed a significant increase of antioxidant potential regarding the controls. Maximum scavenging potential was observed in *A. niger* extracts with SFC. *A. niger* was responsible for the highest increases in extracts scavenging potential in SFC and SBC. A 3.22-fold increase was observed in SFC extracts while in SBC the increment was near 233-fold. Concerning RSC, *A. ibericus* lead to a 21.7-fold increase of scavenging potential.

As shown in **Figure 3E**, SFC fermented extracts reducing power decreased approximally 57 % when compared to control. SFC presented a relatively higher reducing power when compared to RSC and SBC. In this case, the action of the microorganisms may lead to a decrease of electron donor species due to their consumption or lead to the aggregation of species leading to a decrease in the reducing power capacity. On the other hand, regarding RSC and SBC fermented extracts it was observed a clear increase of reducing power ability. Maximum reducing power was observed in fermented extracts of RSC with *R. oryzae* and *A. niger*, showing no statistically significant differences ($p < 0.05$). This is a 20-fold increase while on SBC extracts it was observed a 4.75-fold increase.

SSF was been widely used to improve the nutritional properties of agricultural products and to obtain bioactive compounds. In this work, SSF was able to increase the antioxidant capacity of extracts as previously described by other authors for other substrates. Zhai et al. (2018) reported an increase of 89%, 66.1%, 86.5% and 72.9 % regarding DPPH radical scavenging ability, ferrous ion chelating ability, superoxide anion radical scavenging ability and reducing power, respectively, of ethanolic extracts of corn fermented with *Agaricus brasiliensis* SH26 [73]. The same author reported an increase of 99.3%, 87.4%, 85% and 91.4 % for the same four antioxidant indexes of ethanolic extracts of corn fermented with *A. bisporus* 2796. Xu et al. (2018) tested the effect of SSF on the antioxidant properties of ten cereal grains [74]. This author reported an increase of antioxidant potential of all cereal grains regarding their ability to scavenge the DPPH radical, reducing power ability, ferrous ion chelating activity and superoxide anion radical scavenging capacity of ethanolic extracts. Kang et al. (2016) reported the increase of buckwheat antioxidant potential regarding the DPPH and superoxide scavenging

abilities, reducing power and ferrous ion chelating activity after SSF with *A. blazei* and *A. bisporus* [75].

Nutritional parameters of fermented oil cakes

Relevant physico-chemical parameters to assess nutritional quality of fermented OC were evaluated on fermented and extracted OC. **Figure 4A** shows tin SSF experiments using *A. niger,* no loss of crude protein was found in the fermented OC.

However, with *R. oryzae* and *A. ibericus,* there was a decrease in crude protein during the SSF process. SSF has been used over the past decades to improve the nutritional value of agricultural wastes, namely protein content. In a previous work, SSF of brewer's spent grain, exhausted olive pomace, exhausted grape pomace and vine-shoots trimming was successfully applied for the production of single cell protein, using different *Aspergillus* strains [76]. Canedo et al. (2016) was able to increase crude protein of brewer's spent grain by 2 fold, using the genus *Rhizopus* in SSF [77]. The same fungus was able to increase protein content of rice bran by 58.5 % [78]. However, in this study, the decrease in crude protein content using *R. oryzae* and *A. ibericus* could indicate these fungi use the available N to support their growth and differentiation, as there was not any external N supplementation [79]. Imelda et al. (2008) observed an initial decrease of crude protein during SSF of a mixture of soybean meal, wheat flour, groundnut oil cake and sesamum oil cake (4:3:2:1), when fermented with *A. niger* [80].

During SSF, lignocellulosic fractions are affected by the activity of microorganisms. Filamentous fungi degrade the lignocellulosic matrix into small soluble sugars that can be further consumed by the microorganisms. Compositional changes regarding dietary fibers is quite important during bioconversion studies, such as SSF, once these fractions can affect OC digestibility by animals. NDF and ADF fractions were thus evaluated during this work (**Fig. 4B** and **4C**, respectively). Decrease of these fractions was observed in all fermented OC with the exception of SFC for which NDF and ADF content values of fermented OC were not statistically significant different ($p < 0.05$) from the non-fermented ones. Decreases in cell wall components of 53%, 67% and 69% were observed in SFC, RSC and SBC, respectively, regarding NDF composition. Additionally, regarding ADF decreases of 37%, 63% and 53% were observed for SFC, RSC and SBC respectively. These results may be explained by the action of fungal enzymes produced during SSF that breakdown lignocellulosic bounds between lignin, cellulose and more particularly with hemicellulose [81]. The fiber reduction is an important result of nutritional upgrade of OC for their application as animal feed. The use of filamentous fungi to increase the nutritional value of OC to be used in animal feed does not entail any obstacle and is a viable technique due to the nature of the species used. The fungi used during

this study are recognized as GRAS by the US Food and Drug Administration (FDA). *Aspergillus niger* is one of the most used species in SSF. *A. ibericus* do not produce any relevant toxins and *Rhyzopus oryzae* has been used in SSF studies using agro-food residues for the production of extracellular enzymes [76,82–85].

PCA analysis

A principal component analysis (PCA) was performed in order to assess the relationship between substrates, fungi, total phenolic compounds, antioxidant capacity, enzymes produced, crude and soluble protein and lignocellulosic fractions (NDF and ADF). PCA allowed to obtain a reduced number of lineal combinations of the 13 variables studied to explain data variability. With this analysis, four main components were identified and explain 84.59 % of the variability of original data. The first two principal components, PC1 and PC2 are shown in **Fig. 5** and they accounted for 61 % of the variability (42% and 19% respectively). PC1 was positively characterized by high enzymatic activity (xylanase, cellulase, protease and β-glucosidase), total phenolic compounds (TPC) and antioxidant potential. On the other hand, PC1 was negatively correlated with neutral detergent fiber (NDF) and acid detergent fiber (ADF). As seen in **Fig. 5**, substrates with higher concentrations of lignocellulosic fractions lead to a higher production of xylanase and cellulase. The deconstruction of lignocellulosic materials due to enzymatic activity is also positively correlated with an increased antioxidant potential.

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

Conclusions

This study showed the potential of OC to be used in SSF biotechnological processes to produce enzymes, extraction of antioxidant phenolic compounds and decrease of lignocellulosic fractions of OC in a single process.

Fungi growth led to the production of cellulase, xylanase, β-glucosidase and protease. The production of these enzymes was inducted by the concentrations of cellulose, hemicellulose and proteins in the OC. *A. niger* maximized the production of cellulase, xylanase and protease while *R. oryzae* maximized the production of β-glucosidase and extraction of TPC.

Degradation of lignocellulosic compounds by fungi was demonstrated and this can favor nutrient digestibility of OC by livestock. Also, this degradation led to the release of TPC with potential antioxidant capacity. Again, *A. niger* led to the highest values of antioxidant potential for radicals scavenging, chelation and reduction capabilities. Furthermore, using *A. niger*, fermented OC conserved a high crude protein concentration. Thus, strain MUM 2915 of *A. niger* showed the highest potential for further SSF development and optimization based on OC.

The use of SFC and RSC for animal feed could be a step further to decrease soy production for animal feed, leading to the increase of arable land for other crops and diminishing the pressure on natural resources.

In conclusion, all OC proved to be suitable substrates for SSF, a biotechnological process that did not generate any additional waste. SSF was successfully applied to obtain new fermented and upgraded OC as well as enzymatic extracts with antioxidant capacity, that also have potential to be applied in the food and feed industry.

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FIGURE CAPTIONS

Figure 1. Total phenolic content (TPC) and antioxidant potential of aqueous extracts of non-fermented oilseed cakes. **(A**) TPC; (**B**) DPPH radical scavenging activity; (**C**) iron chelation ability; **(D)** superoxide radical scavenging activity; **(E)** reducing ability. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters are not statistically significant different (Tukey test; $P< 0.05$).

Figure 2. Cellulases (**A**), xylanases (**B**), β-glucosidases (**C**) and proteases **(D)** production during SSF. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters are not statistically significant different (Tukey test; $P < 0.05$). Statistical analysis is relative to each oilseed cake.

Figure 3. Total phenolic content (TPC) and antioxidant potential of aqueous extracts of controls (oilseed cake sterilized) and fermented oilseed cakes. **(A**) TPC; (**B**) DPPH radical scavenging activity; (**C**) iron chelation ability; **(D)** superoxide radical scavenging activity; **(E)** reducing ability. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters are not statistically significant different (Tukey test; $P < 0.05$). Statistical analysis is relative to each oilseed cake.

Figure 4. Crude protein (**A**), neutral detergent fiber (NDF) (**B**) and acid detergent fiber (ADF) (**C**) of controls (oilseed cake sterilized) and fermented oilseed cakes. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters are not statistically significant different (Tukey test; $P< 0.05$). Statistical analysis is relative to each oilseed cake.

Figure 5. Principle component analysis. Biplot representation of variables and SSF experiments performed. CTRL, control; RO, *R. oryzae*; AI, *A. ibericus*; AN, *A. niger*.

TABLES

Table 1. Composition of oilseed cakes (% w/w) and chemical composition of oilseed cakes reported in literature.

Parameter	SFC		RSC		SBC	
	Experimental	Literature	Experimental	Literature	Experimental	Literature
Humidity	9.82 ± 0.09	5.56 – 7.60 [1, 2, 3]	15.26 ± 0.30	8.02 – 10.10 [2, 4]	13.61 ± 0.16	9.3 – 9.92 [4, 5, 6]
Ash	8.38 ± 0.01	5.26 – 7.50 [1, 2, 7, 8]	6.66 ± 0.22	5.6 - 7.0 [2, 4, 7, 9]	6.26 ± 0.10	5.83 – 7.48 [4, 5, 6, 7, 10]
Protein	40.20 ± 0.59	23.60 – 52.50 [1, 2, 3, 7, 8]	39.83 ± 2.09	33.90 -40.30 [2, 7, 9, 11]	50.41 ± 1.43	40.20 – 49.30 [4, 7, 10]
Nitrogen	6.95 ± 0.51	4.70 [2]	7.64 ± 0.70	7.5 [2]	9.11 ± 0.30	8.21 [5]
Carbon	46.66 ± 0.23	49.80 [2]	50.59 ± 0.81	50.20 [2]	47.35 ± 0.49	44.80 [5]
C/N ratio	6.71	9.8 – 10.60 [2, 12]	6.62	6.69 – 8.40 [2, 12]	5.20	5.46 [5]
Lipids	2.34 ± 0.39	2.75 – 30.30 [1, 2, 3]	2.04 ± 0.28	11.80 – 16.70 [2, 9]	1.72 ± 0.16	2.1 [6]
Water Holding capacity	359 ± 35		193 ± 5		285 ± 30	500 [13]
pH [a]	5.33		5.50		5.22	
Klason lignin	8.25 ± 0.05	7.72 – 13.30 [2, 14, 15]	7.82 ± 2.25	6-53 – 9.00 [2, 15]	2.67 ± 1.39	2.80 [5]
Cellulose	14.28 ± 0.24	12.30 – 25.10 [2, 14, 15]	15.47 ± 1.64	5.90 – 8.18 [2, 15]	16.15 ± 0.27	21.58 [5]
Hemicellulose	11.14 ± 0.67	4.42 – 15.40 [2, 14]	13.63 ± 1.43	2.04 [2, 15]	15.55 ± 0.65	52.51[5]
NDF	33.68	30.29 – 41.10 [16, 17]	36.84	29.90 [18]	33.91	11.10 – 15.89 [18]
ADF	22.53	19.92 – 32.63 [16, 17]	23.21		18.36	8.41
Soluble protein [b]	0.79 ± 0.04		1.19 ± 0.07		0.84 ± 0.02	
Free sugars [b]	0.77 ± 0.01		0.97 ± 0.05		0.73 ± 0.03	
Total phenolic compounds [b]	1.17 ± 0.02		0.48 ± 0.02		0.31 ± 0.02	

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

[a] Measured on the solid after aqueous extraction

[b] Quantified in aqueous extracts

Reference	Number
[86]	1
[33]	2
[87]	3
[88]	4
[35]	5
[89]	6
[90]	7
[91]	8
[92]	9
[93]	10
[94]	11
[34]	12
[40]	13
[95]	14
[96]	15
[97]	16
[98]	17
[88]	18
[99]	19

FIGURES

Figure 1:

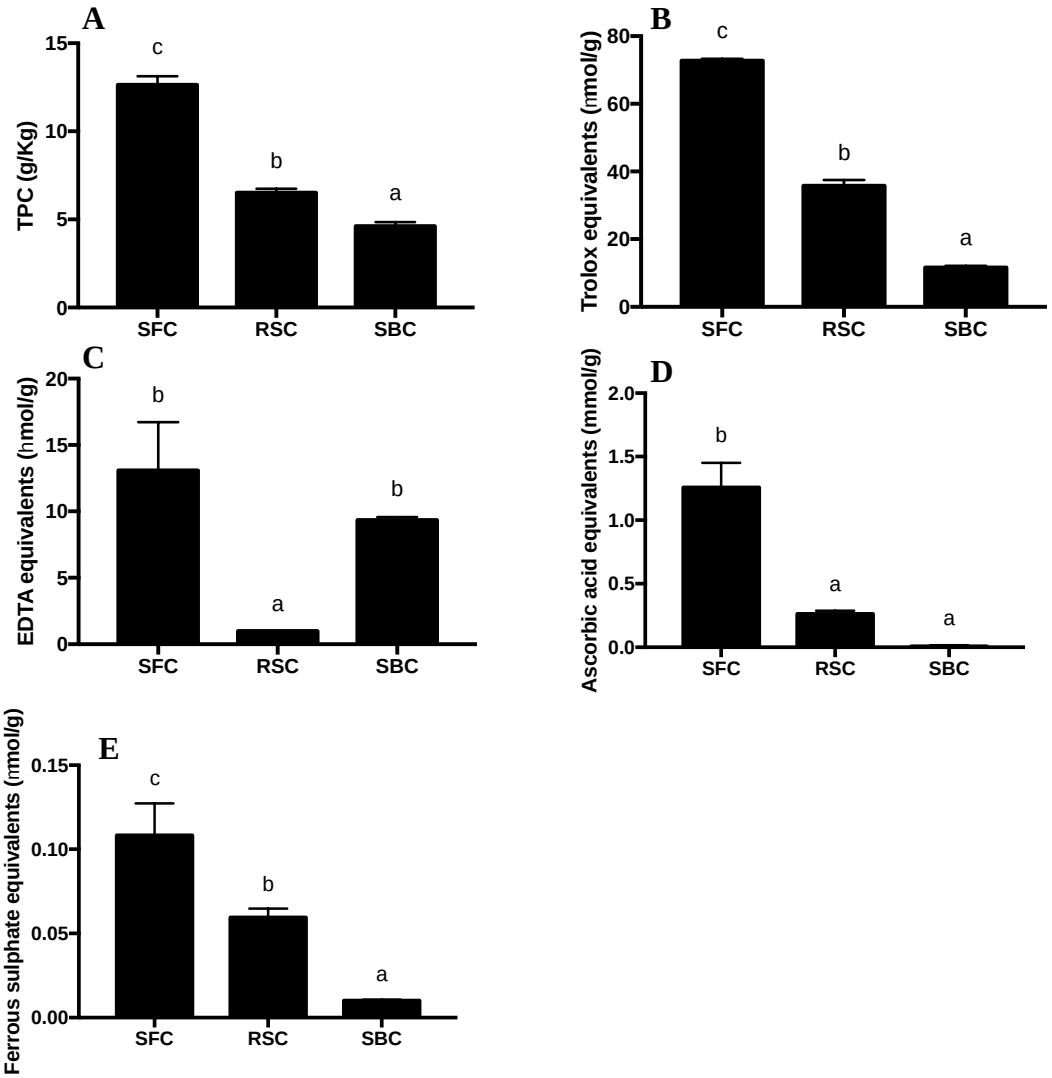
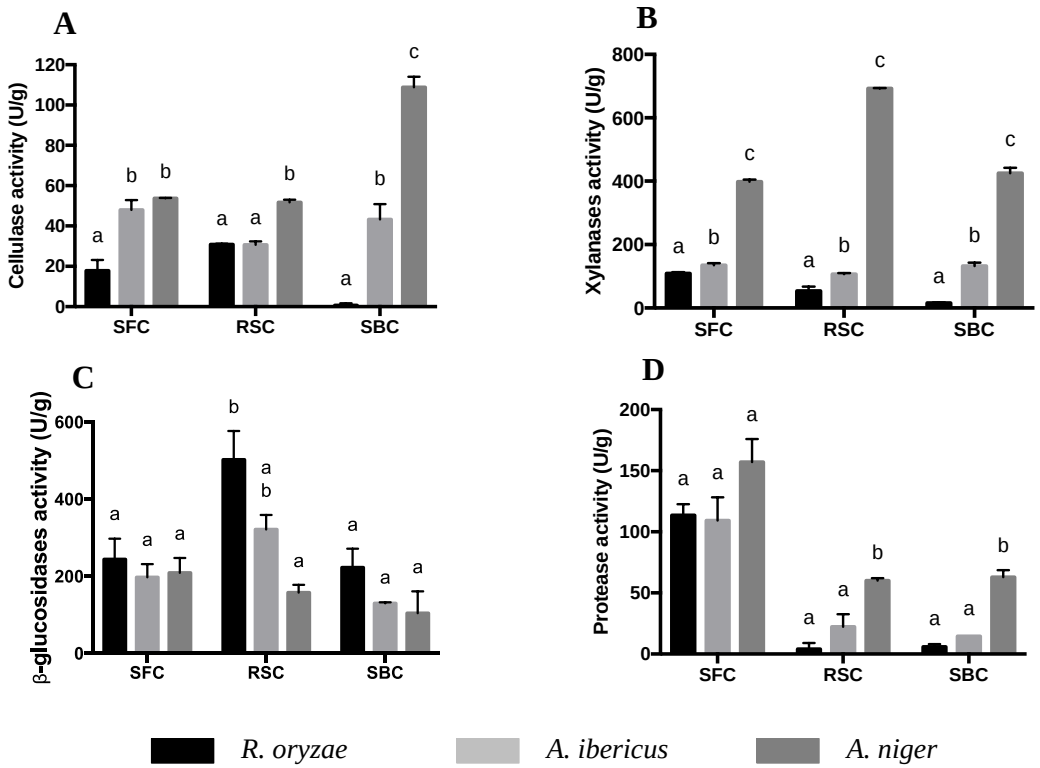


Figure 2:


A
Cellulase activity (U/g)
120
100
80
60
40
20
0
a
b
b
a
a
b
a
b
c
SFC
RSC
SBC
B
Xylanases activity (U/g)
800
600
400
200
0
a
b
c
a
b
c
a
b
c
SFC
RSC
SBC
C
β-glucosidases activity (U/g)
600
400
200
0
a
a
a
b
a
b
a
a
a
SFC
RSC
SBC
D
Protease activity (U/g)
200
150
100
50
0
a
a
a
a
a
b
a
a
b
SFC
RSC
SBC
R. oryzae
A. ibericus
A. niger

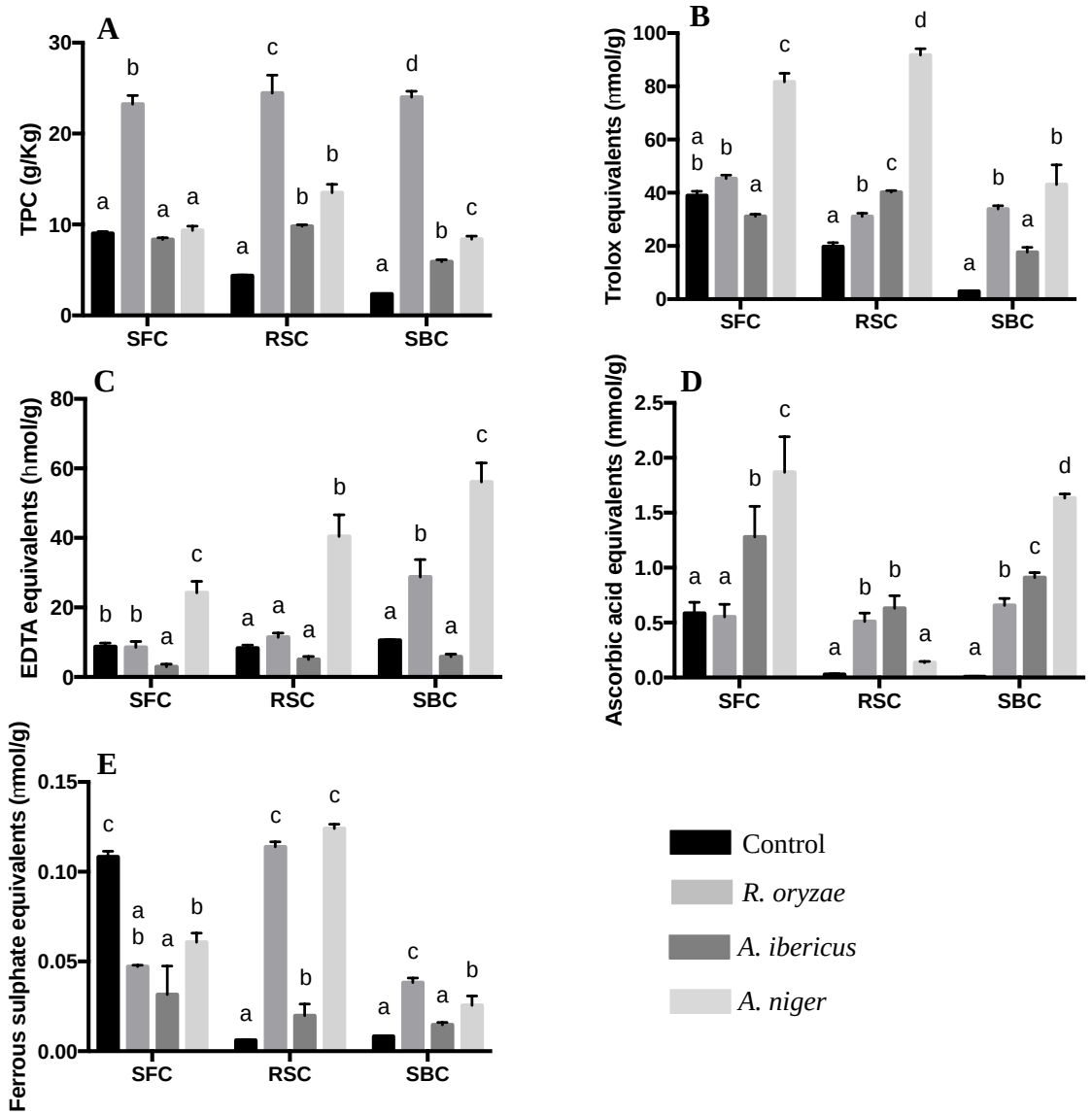

A
TPC (g/Kg)
30
20
10
0
SFC
RSC
SBC
B
Trolox equivalents (mmol/g)
100
80
60
40
20
0
C
EDTA equivalents (hmol/g)
80
60
40
20
0
D
Ascorbic acid equivalents (mmol/g)
2.5
2.0
1.5
1.0
0.5
0.0
E
Ferrous sulphate equivalents (mmol/g)
0.15
0.10
0.05
0.00
Control
R. oryzae
A. ibericus
A. niger

Figure 3:

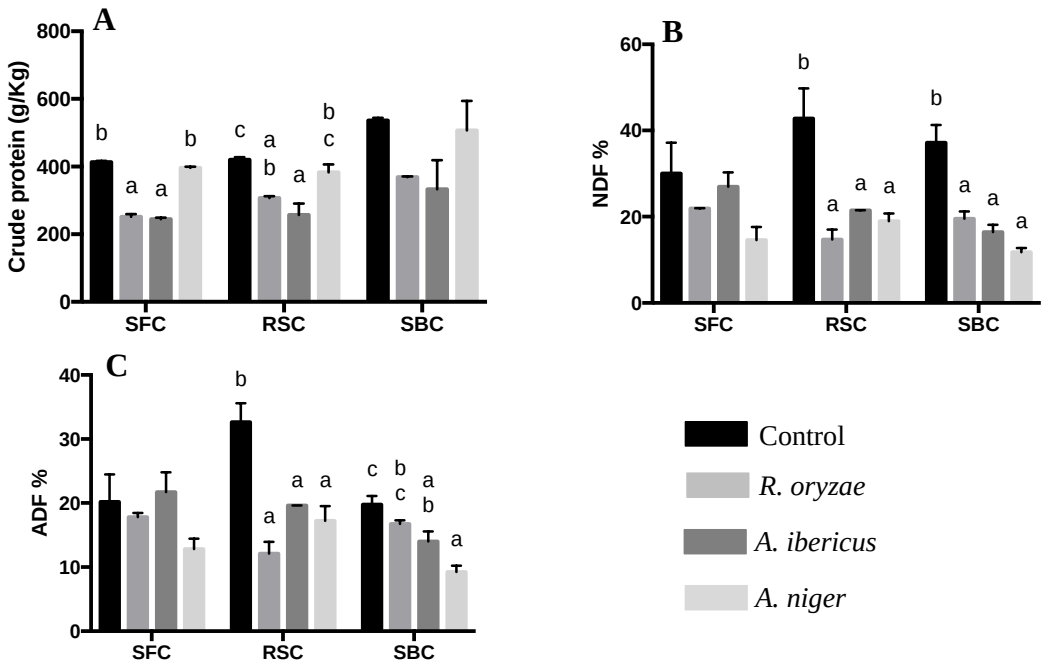


Figure 4:

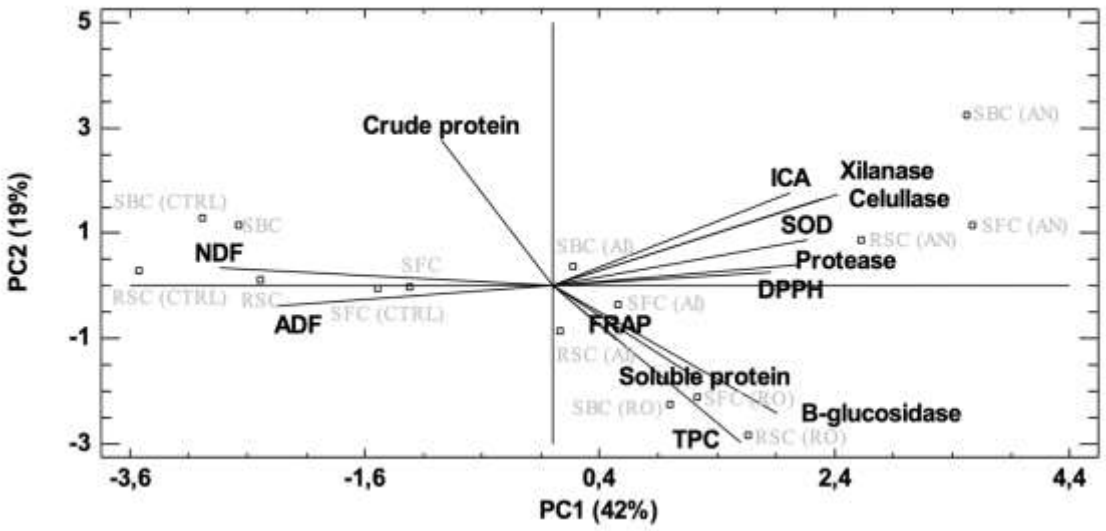


Figure 5: