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

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

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Abstract

Sunflower (SFC), rapeseed (RSC), and soybean (SBC) cakes are examples of oilseed cakes (OC) used in animal feed. Bioprocessing of these OC by solid-state fermentation (SSF) aims to boost OC applications in feed and other industries by reducing antinutritional factors and releasing enzymes and antioxidants.

A simplex centroid design was performed to optimize the substrate composition of SFC, RSC and SBC mixtures that maximize lignocellulolytic enzymes production by SSF with *Aspergillus niger* and, consequently improve the nutritional properties of OC. Enzymes production by SSF of OC mixtures exceeded the activity values obtained using single OC. A mixture composed by 50 % (w/w) RSC and SBC was found as the optimum

substrate. A scale-up from flasks to tray-type bioreactors demonstrated SSF reproducibility leading to the production of 299 U g^{-1} cellulase, 1476 U g^{-1} xylanase, 191 U g^{-1} β-glucosidase, and 220 U g^{-1} protease. The obtained enzymatic extract also presented antioxidant activity (around 50 μmol Trolox equivalents/g). This study demonstrated that bioprocessing of OC mixtures through SSF is an effective approach to obtain value-added products with applications as feed or feed additive and to increase the liberation of bioactive compounds with applications in the feed, food, cosmetic or pharmaceutical industries.

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

1. Introduction

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

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

Soybean is the largest produced oilseed on a global scale being the United States, Brazil, and Argentina the major world producers. In 2021, roughly 373 million tons of this commodity were produced, and projections show that its products will continue to rise, with the possibility to achieve approximately 411 million tons by 2030.[1] However,

concerns with soy production sustainability have arose over the last years due to intensive production which is related with non-rotation crops, deforestation, and loss of native vegetation. According to Fraanje and Garnett,[2] nearly 75 % of produced soybean is used to feed animals, being the poultry sector the major consumer of processed soybean (53 %) amongst all livestock and poultry species. According to the Organization for the Economic Co-operation and Development and the Food and Agriculture Organization (OECD/FAO) Agriculture Outlook for 2021-2030, production of rapeseed, sunflower seed, and groundnuts will continue to rise. These crops are among the largest produced, after soybean. In 2021, around 159 million tons of these seeds were produced, and by 2030 the forecast is to achieve roughly 179 million tons.[1]

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

OC are characterized by a high protein content, the presence of non-starch polysaccharides, fibers, antioxidants, lipids, minerals, and vitamins.[6,7] Independently of the use of organic solvents after mechanical extraction of the vegetable oils, some polar compounds such as phenolic acids, lignans, or flavonoids with antioxidant potential

remain in the OC.[8] These bioactive compounds can be extracted, concentrated, and incorporated in foods and feed as additives providing additional protection against oxidative stress. The protein content of sunflower cake (SFC), rapeseed cake (RSC), and soybean cake (SBC) account for 14-46 % (w/w) of dry weight while fiber varies between 5 and 37 %.[9,10] Additionally, OC have residual concentrations of phenolic compounds ranging from 3 to 12 g kg^{-1} with antioxidant properties allowing them to act as radical scavengers, chelators, or reduction agents.[9] The employment of these OC in low-cost and environmental impact technologies such as bioprocess allows the valorization of this biomass contributing to the improvement of vegetable oils production chains.

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

Filamentous fungi are natural degraders of lignocellulosic biomass as they produce extracellular lignocellulolytic enzymes.[17] Additionally, they can develop hyphae that penetrate the solid matrix of substrates. The depolymerization of lignocellulosic biomass

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

This work aimed to study the bioprocessing of mixtures of OC for obtaining value-added products in the concept of biorefinery contributing to the implementation of the circular economy. In this sense, different mixtures of OC were evaluated as substrate for SSF to produce cellulase, xylanase, β-glucosidase, and protease and concomitantly liberate phenolic compounds with antioxidant potential. Additionally, the effect of SSF on substrate composition (crude protein and fiber) was evaluated. To assess the optimum mixture of substrates, a simplex centroid mixture design was used. The use of statistical mixture designs has the advantage of a clear representation of effects and interactions between different components in a certain response.[20] SSF at optimal conditions was scaled-up to tray-type bioreactor.

2. Materials and methods

2.1. Agro-food by-products

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

2.2. Microorganisms

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

2.3. Solid-state fermentation

The mixture of substrates was performed according to **Table 1**, following a simplex centroid mixture design. SSF of each experiment was carried out in 500 mL Erlenmeyer flasks with cotton caps to allow oxygen transfer. A total amount of 10 g of dry solid was used, moisture was adjusted to 75 % with distilled water (w/w, wet basis). Flasks were sterilized for 15 min at 121 ºC. For inoculation of *A. niger* into the different substrate

mixtures, a sterile solution of peptone composed of 1 g L^{-1} peptone and 0.1 g L^{-1} Tween-80 was used to recover spores from PDA slants and to prepare an inoculum suspension with a concentration of 10^6 spores mL^{-1}, from which 2 mL was added to each flask. The SSF was carried out over 7 days at 25 ºC in a controlled temperature chamber. Each experiment was performed in duplicate and a control experiment without inoculation was performed following the SSF conditions described.

2.4. Bioactive compounds extraction

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

2.5. Enzymatic activities

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

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

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

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

2.6. Chemical composition and nutritional parameters

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

Nitrogen content of solid residues was quantified using the Kjeldahl method and estimation of crude protein was obtained using a defined factor of 6.25.[21]

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

2.7. Total phenolic compounds and antioxidant activity

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

The antioxidant potential of fermented extracts was evaluated using four *in vitro* antioxidant assays as described by Sousa et al.[9] The scavenging potential of free radicals was evaluated by the DPPH method and expressed as micromoles of Trolox equivalents per gram of dry matter (µmol g^{-1}).[23] Extract's capacity to chelate ferrous ion was assessed by the iron chelating ability (ICA) assay and expressed as EDTA equivalents (nmol/g).

Scavenging of superoxide radical was determined using the PMS-NADH non-enzymatic assay and expressed as micromoles of ascorbic acid equivalents per gram of dry matter (μmol g^{-1}).[24] Extracts reduction potential was evaluated by the Ferric Reducing Antioxidant Power Assay (FRAP) and expressed as ferrous sulfate equivalents (μmol/g).[25] All assays were performed in duplicate.

2.8. Experimental design and statistical analysis

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

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

$$Y = b1.x1 + b2.x2 + b3.x3 + b12.x1.x2 + b13.x1.x3 + b23.x2.x3 + b123.x1.x2.x3$$

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

2.9. Scale-up process to tray-type bioreactors

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

3. Results and discussion

3.1. Chemical composition of oilseed cakes and their mixtures

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

substrate combinations of OC (**Table 1**) were selected, to evaluate their impact on microbial extracellular enzymes (cellulase, xylanase, β-glucosidase, and protease) production and to obtain antioxidant potentially rich extracts. SBC has the highest crude protein content, being 1.3-fold higher than in the other two OC. Also, SBC has the highest cellulose and hemicellulose content. On the other hand, lignin content was highest in RSC and lowest in SBC. Lignin concentration of RSC was 3 and 6-fold higher than of SFC and SBC, respectively. The mixtures of SBC with the RSC and SFC, presented balanced composition of fibers and protein that may result in improved performance of the SSF of OC by the fungus. **Table 2** shows the results of the dependent variables obtained in each SSF with *A. niger*, according with the designed runs of the experimental matrix described in **Table 1**.

The mixture of OC had a significant impact on the production of all enzymes resulting in increased production, compared to the use of single OC as substrate. Cellulase activity obtained in mixtures of two different OCs was around 3-fold higher than the obtained by SSF of single SBC and 6-fold higher than the obtained with single SFC and RSC. Mixtures of the three OCs led to the production of 2-fold more cellulase than with single SBC that was the single best substrate for cellulase production, which is in accordance with the highest content of cellulose of this OC. As regards other enzymes (xylanase, β-glucosidase, and protease), **Table 2** shows that the mixture of all OCs (run 7) led to the best activity values, showing the synergistic effects of OCs in the solid medium composition that allowed best performance of the fungus. Closely to these results, are the obtained in SSF of run 6 composed by a mixture of 50 % (w/w) of RSC and SBC, particularly for xylanase and protease. As a result of the enzymes production through SSF, the solid substrate composition suffered modifications and the lowest values of cellulose, hemicellulose and lignin content in fermented OCs mixtures was obtained in

run 6 with the mixture of RSC and SBC. Despite being observed a lignin concentration in this fermented mixture when compared to the corresponding unfermented mixture (**Table 1**), the fermented conjugation of RSC and SBC showed a lower content of lignin compared to the corresponding fermented single OC's.

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

3.1.1. Optimum conditions predicted for enzymes production

This experimental design allowed the identification of effects of each OC and their interactions on enzymes production. Additionally, it was possible to find the optimum mixtures of OCs that maximizes the enzymatic activities obtained after SSF. The regression coefficients and statistical parameters of the experimental design are shown in **Table 3**. The statistical parameters show a good fit of the models supported by the coefficient of determination (R^2) and F-value. The coefficient of determination was above 0.96 for all the enzymes except for β-glucosidase (R^2 of 0.74). These coefficients validate the experimental data, meaning they agree with the predicted ones by the model. Additionally, apart from β-glucosidase, all enzymes showed high F-values indicating that the models were statistically significant ($P < 0.05$). The positive regression coefficients obtained for most of the independent variables represent the synergistic interactions while negative values indicate that the components in the mixture act antagonistically. Despite the type of interaction in each substrate mixture, not all the combinations have a statistically significant effect on the dependent variables' response. Statistically significant effects ($P < 0.001$ and $P < 0.01$) were observed for every independent variable considering cellulase and xylanase production. For β-glucosidase, the mixture of OC had

no effects and only the single substrates x_1, x_2 and x_3 had significant effects ($P < 0.001$ and $P < 0.01$) on this enzyme production. Except for the ternary combination of OC ($x_1x_2x_3$), all the substrate compositions had positive effects on protease production. The coefficients of x_1, x_2, x_3 and x_2x_3 had high significant effect ($P < 0.001$) on this enzyme production, followed by x_1x_2 ($P < 0.01$) and finally by x_1x_3 ($P < 0.05$).

The mixture contour plots represented in **Figure 1** show the variation of enzyme activity using different amounts of OC. Each plot represents a dependent variable, and the corner of each triangle represents the OC used as substrates. The surface of the triangle shows changes in substrate composition and the correspondent enzyme production predicted by the model. The darker zone of each triangle represents the optimum mixture of substrates that maximize each dependent variable.

As illustrated in **Figure 1a**, maximum cellulase production was obtained by the binary combinations of OC as discussed above. As shown in **Figure 1b** by the counterplot of xylanase, the darker zone is focused on the center of the triangle but slightly directed to the mixture of RSC and SBC. The mixture of the three OC in equal proportions (run 7) favored the production of β-glucosidase. However, the lower value of the coefficient of determination for this variable, indicates a non-satisfactory model fit. The optimum mixture for protease production was obtained by the combination, in equal proportions, of RSC and SBC. Protease production was increased by 3.7-fold compared to the use of RSC or SBC as single substrates.

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

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

3.1.2. Optimum conditions predicted for nutritional parameters

The chemical composition of OC was evaluated after SSF to understand the effect of enzymes production on lignocellulosic fractions and crude protein (CP). CP was not significantly affected by SSF. OC are natural sources of vegetal protein, and its protein content ranges from 40 to 50 % (w/w) on a dry matter basis, remaining within the same ranges after SSF. On the other hand, hemicellulose and cellulose fractions were strongly affected by SSF. A significant decrease of these fractions was observed in every run of the experimental design. The hemicellulose reduction obtained by SSF of OC mixtures

ranged from 47 % (w/w) to 71 %, but cellulose reduction was lower than the observed for hemicellulose, as this component comprises the interior part of the cell wall and presents a crystalline structure. Even though, reductions of 47 % were observed in runs 2 and 3 while the lowest (18 %) was obtained in run 4. After SSF, there was an increased concentration of lignin in every run. The highest value was observed in run 3 representing a lignin concentration nearly 12-fold of the unfermented SBC. The high values of lignin obtained after SSF can be explained by the concentrating effect of SSF as a consequence of the high reduction of hemicellulose and cellulose. In fact, the absolute lignin mass in the solid, did not significantly change since there was a solubilization of around half of the initial mass of the substrate mostly from the other polysaccharides than lignin. Thus, these soluble compounds were removed during the extraction process leading to a concentration of lignin in the final solid. The coefficients of determination of CP, cellulose, and hemicellulose ranged from 0.67 to 0.73. The statistical parameters obtained for these variables indicate that there is not a good fit by the model to these single dependent variables. As so, no valid conclusions can be outlined regarding the effect of OC mixtures on the crude protein and lignocellulosic composition (cellulose and hemicellulose). On the other hand, for lignin content a coefficient of determination of 0.88 and an F-value of 8.3 were obtained, validating the experimental data fitting to the model. The coefficients of regression indicate that the mixture of OC did not have effects in these dependent variables. Significant effects ($P < 0.001$) of x_1, x_2 and x_3 were observed for CP and for lignin ($P < 0.001$ and $P < 0.01$). For hemicellulose content only x_2 ($P < 0.05$) and x_3 ($P < 0.001$) showed significant effects. The mixture contour plots indicate that the presence of SBC as substrate in SSF led to the best result in terms of highest CP and the lowest lignin content (**Fig. 1i** and **h**). On the other hand, the absence of SBC in the substrate mixture of SFC and RSC, favored the decrease of cellulose and

hemicellulose (**Fig. 2 j)** and **k)**). Other studies evaluated the use of SSF to decrease the fibers content of lignocellulosic materials by other fungi. Xu et al. reported a decrease of cellulose (23.5 %), hemicellulose (11.7 %), and lignin (22.4 %) in raw sugarcane bagasse, after 48 h of hydrolysis using a cellulase cocktail obtained after 6 days of SSF of wheat bran with *Inonotus obliquus*.[30] The same author reported decreases of cellulose (18.9 %), hemicellulose (11.2 %), and lignin (14.8 %) for rice straw using the same enzymatic cocktail in saccharification processing. Sousa et al. reported decreases of cellulose, hemicellulose, and lignin after SSF of brewer's spent grain, exhausted olive pomace, exhausted grape marc, and vineshoot trimmings, using independently three different *Aspergillus* species.[12]

3.2. Global optimization by multiple response variable

The maximization of multiple variables is a key step to obtain an optimized substrate able to produce a broad range of bioactive compounds with multiple applications via SSF. **Table 4** shows the optimal mixture of OC that maximizes the production of enzymes and CP while minimizing cellulose, hemicellulose, and lignin concentration. The composition of the optimum substrate is comparable to run 6 (**Table 1**) and the predicted values of the dependent variables, by the model are within the range of the experimental values obtained in runs 6 and 7 (**Table 2**). These combinations of OC with optimized chemical composition and simultaneous production of carbohydrases may constitute a value-added product to be applied as animal feed.

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

The effect of substrate composition and SSF on TPC release and antioxidant potential of aqueous extracts of the optimal mixtures of OC was also evaluated (**Fig. 2**).

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

As observed in **Fig. 2 B-E** the antioxidant potential of aqueous extracts was increased by SSF except for the iron chelation potential (**Fig. 2C**).

The scavenging potential of free radicals (**Fig. 2B**) was increased approximately by 4.6-fold for run 6 while the potential of the ternary mixture was increased by 2.4-fold, compared to respective controls. The scavenging of superoxide radicals (**Fig. 2D**) and extracts reduction potential (**Fig. 2E**) followed the same pattern with increases in the range of approximately 40-fold and 4-fold and 21-fold and 2.4-fold for runs 6 and 7, respectively. On the other hand, iron chelation potential decreased 2.5-fold and 1.7-fold for run 6 and 7, respectively.

The extracellular enzymes produced during SSF are involved in the release of phenolic compounds bounded to the lignocellulosic matrix of OC. Additionally, these enzymes may alter the chemical structures of phenolic compounds during SSF which would

influence the antioxidant potential of samples. The enhanced antioxidant potential can be attributed to the formation of phenolic aglycones as a consequence of deglycosylation by carbohydrases.[34,35]

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

3.4. Scale-up for tray bioreactor validation

Due to the experimental data similarity observed between runs 6 and 7, these optimum mixtures of OC were selected for scaling-up to tray-type bioreactor. **Table 5** depicts the results obtained after the scale-up process for the studied dependent variables. Punctual variations were observed on the dependent variables' responses. Statistically significant differences ($P < 0.05$) were observed in β-glucosidase activity for run 6, considering the use of Erlenmeyer flask and small tray bioreactor. However, considering the same enzymatic activity, experimental results obtained using 1 L Erlenmeyer flasks containing 50 g of substrate were successfully reproduced at a larger scale (400 g tray bioreactor). The antioxidant potential was also evaluated through the scavenging potential of free radicals (DPPH). The results indicate that no statistically significant differences were observed for this bioactivity proving its reproducibility at different scales of the process. The same phenomenon regarding antioxidant potential and enzymatic activity was observed for the scale-up of run 7. The crude protein and lignocellulosic composition of

fermented OC showed slight variations between experiments during the scale-up process but without statistical significance.

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

4. Conclusions

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

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

Conflict of interest

The authors confirm that they have no conflicts of interest for the work described in this manuscript.

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FIGURE CAPTIONS

Figure 1- Contour plots of each dependent variable obtained by the simplex centroid design after SSF with *A. niger*: a) cellulase; b) xylanase; c) b-glucosidase; d) protease; e) crude protein; f) cellulose; g) hemicellulose; h) lignin.

Figure 2- Total phenolic content (TPC) and antioxidant potential of aqueous extracts of the optimum mixtures of fermented oilseed cakes ( ) with *A. niger*. Control ( ) represents the autoclaved substrates without inoculation and the SSF. (A) TPC; (B) DPPH radical scavenging activity; (C) iron chelation ability; (D) superoxide radical scavenging activity; (E) reducing ability. Results represent the average of two independent experiments and error bars represent standard deviation. Bars with equal letters for the same run are not statistically significant different (Tukey test; $P < 0.05$).

TABLES

Table 1- Characterization of the solid substrate of each experiment of the simplex centroid mixture design.

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

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

Table 2- Composition of solid substrates after solid-state fermentation and results of studied dependent variables using simplex centroid design.

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

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

Table 3- Statistical parameters of simplex centroid mixture design.

RC	Xyl	Cel	β-gluc	Prot	CP	Cellulose	Hemicellulose	Lignin
x_1	398.27***	53.76***	209.31***	157.11***	396.28***	-5.32	-6.42	10.07**
x_2	692.02***	51.74***	158.23***	60.08***	382.69***	-5.30	-6.58*	7.52**
x_3	424.79***	108.90***	104.51**	62.95***	507.03***	-6.54	-10.98**	11.59***
x_1x_2	1650.38***	962.48***	26.98	190.59**	-25.00	19.62	7.11	-13.57
x_1x_3	2698.95***	842.60***	187.22	129.09*	-37.95	11.56	8.73	-8.81
x_2x_3	3668.46***	875.06***	239.72	625.01***	113.04	3.74	-2.90	-8.95
$x_1x_2x_3$	4203.58**	-4272***	815.21	-178.93	534.33	-34.90	-5.21	80.75
Model (SS)	2605850	155247	22027	43198.5	27927.6	39.89	23.85	219.14
Total error (SS)	10051	683.43	7614.89	1605.23	10111.6	19.33	9.42	30.81
R^2	0,996	0,996	0,743	0,964	3	0,674	0,717	0,877
R^2 adjusted	0,993	0,992	0,523	0,934	0,506	0.394	0.474	0,771
F-value	302.47	265.02	3.37	31.40	3.22	2.41	2.96	8.30

RC, regression coefficients; R^2, coefficient of determination; Cel, cellulase; Xyl, xylanase; β-glu, b-glucosidase; Pro, protease; CP, crude protein.
***P < 0.001; ** P < 0.01; * P < 0.05

Table 4- Optimization by multiple response variables of enzymes and nutritional properties.

Optimization	Mixture composition (%)			Dependent variables (predicted value)							
	SFC	RSC	SBC	Xyl (U g^{-1})	Cel (U g^{-1})	B-glu. (U g-1)	Prot. (U g^{-1})	C.P. (g Kg^{-1})	Cellulose (g Kg^{-1})	Hemicellulose (g Kg^{-1})	Lignin (g Kg^{-1})
Enzymes and nutritional properties	0	47	53	1466	300	190	217	476	100	40	180

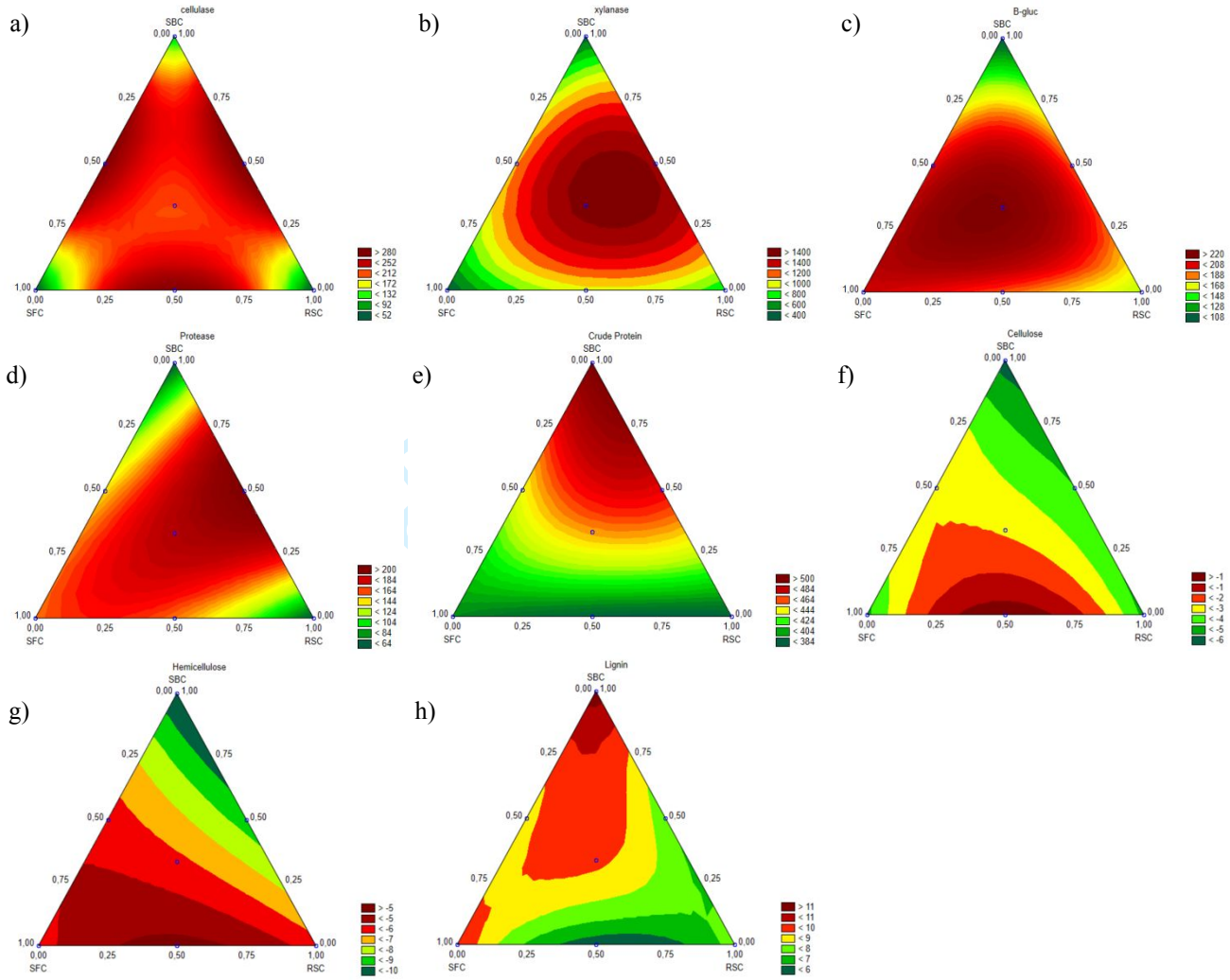
Table 5- Scale-up of run 6 using 50 g and 400 g of dry substrate in 1L Erlenmeyer flask and tray bioreactors

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

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

1 **FIGURES**

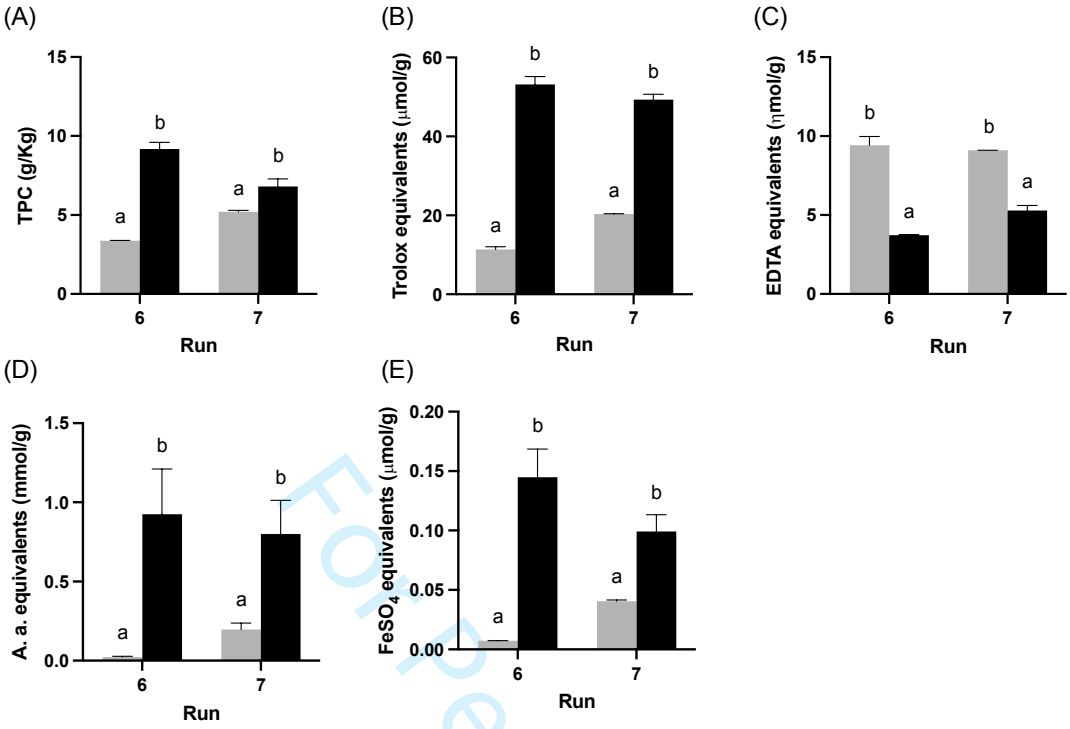
2 **Figure 1:**



3

4

Figure 2:



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