Research Article

Lipase production by solid-state fermentation of olive pomace in tray-type and pressurized bioreactors

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Abstract

BACKGROUND: Bioreactor type, sterilization and specific operational conditions are key factors for the scale-up of solid-state fermentation (SSF). This work deals with the lipase production by SSF of olive pomace (OP) in traditional tray-type and pressurized bioreactors. Important aspects of SSF in bioreactors were studied, such as the need for sterilization and moisture content (MC) control.

RESULTS: At larger scale, there was no significant difference in lipase production between sterilized and unsterilized substrates, but MC control had significant impact. The production of lipase in a pressurized bioreactor, under air absolute pressure of 200 kPa and 400 kPa, was 2-fold higher than in a tray-type bioreactor using the same amount of substrate (500 g) and the same bed height. The protein content of substrate increased from 10 to 18% (w/w) after SSF and the fermented solid presented an antioxidant activity of 10 mmol Trolox kg^{-1}.

CONCLUSIONS: SSF in a pressurized bioreactor enabled efficient production of lipase with higher substrate bed height in contrast to that in a tray-type bioreactor. The improvement in nutritional value of the substrate by SSF indicates its potential applicability in animal feed.
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Keywords: solid-state fermentation; olive pomace; lipase production; tray-type bioreactor; pressurized bioreactor

INTRODUCTION

Solid-state fermentation (SSF) is defined as a fermentation process involving a moistened solid substrate as a support for growth and metabolism of microorganisms.[1,2] SSF simulates the natural habitat of microorganisms grown on moist substrates, while submerged fermentation (SmF) can be considered as a change in their natural habitat, especially for filamentous fungi.[1] The sterilization of substrate is a challenge in the scale-up of SSF to industrial development. The difficulty of filling the bioreactors with solid substrate in sterile conditions makes it essential to use sterile-in-place bioreactors, which increases the process costs (technology, time and energy consumption). Due to low water activity in SSF, sometimes the sterilization may not be needed.[2] On the other hand, SSF offers the opportunity of using low cost agro-industrial residues for metabolite production.[1]

One such low cost residue is olive pomace (OP). OP is a sludgy waste generated by the two-phase extraction system of olive oil. It is an acidic and very moist material (74% (w/w), wet basis), rich in organic matter, it contains water-soluble carbohydrates, phenols and residual olive oil,[3] offering an excellent source of nutrients to produce lipases, since the remaining oil induces its production by microorganisms. In fact, previous work has demonstrated the ability of *Aspergillus ibericus* MUM 03.49 to produce lipase[4] by SSF of OP with wheat bran (WB), and by SmF of olive mill wastewaters.[5] The demand for lipases is increasing

and its market was projected to reach $590.5 million by 2020.[6] Lipases have many applications as additives in the food industry, fine chemicals, detergents, wastewater treatment, cosmetics, pharmaceuticals, leather processing and biomedical assays.[7]

In SSF processes at industrial level, the growth of microorganism and the production of metabolites may be limited by heat, nutrients and oxygen transfer. The design of bioreactor can help to reduce these limitations.[8] Different bioreactor types have been used in SSF, including tray-type, packed-bed and horizontal rotary-drum, each one having advantages and disadvantages.[2] A tray-type bioreactor consists of a chamber in which air, with controlled temperature and relative humidity, circulates around the trays. Each tray contains a thin layer of substrate, and usually has an open top and a perforated bottom. In addition, the relative humidity should be high to avoid the drying of the bed surface.[8]

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If fermentations are undertaken in trays with increasing bed thickness, the mass and energy transfer become more difficult. In this sense, the metabolic heat produced during the fermentation process is accumulated in the substrate bed, and the difficulty of transferring the heat from the centre of the bed can create gradients of temperature.[9] Furthermore, the mycelium growth during the fermentation period accumulates in the substrate bed, filling up void spaces between particles, hindering the diffusion of air inside the substrate.[9] Scale-up may be achieved by increasing the area of the trays, using wider trays, or simply using more trays.[10] To avoid/or decrease drying of the substrate bed in SSF performed in a tray-type bioreactor, the substrate bed height and moist air supply must be considered.[11]

In rotary-drum bioreactors, rotation is applied to improve mass and heat transfer; however, the shearing forces may disrupt fungus mycelium.[12] A pressurized bioreactor may overcome some of the previous problems, since the increased air pressure improves the oxygen transfer rate from air to aerobic cultures, increasing oxygen solubility in the media.[13] This presents important operating cost savings in power consumption, that compensates the additional capital cost investment of the pressurized bioreactor.[14,15] The pressurized bioreactor is typically a tank bioreactor already used in industry, such as in chemical processes, and may be constructed at several scales. However, there are limits for application of increased air pressure and consequently of increased oxygen partial pressure, since above certain values toxic effects on cell activity and product formation may occur.[16] Increased air pressure has been successfully used in SmF, as reported by Lopes *et al.*,[17,18] who obtained significant improvements in lipase productivity by *Yarrowia lipolytica* under SmF, using air pressure up to 600 kPa. The use of continuous pressurized aeration in SSF has not been reported; however, pulse air pressure increases have been used to enhance mass and heat transfer in SSF, stimulating the growth and metabolism of microorganisms,[19] as well as the production of different enzymes.[20–23]

SSF of agro-industrial residues is a promising alternative to improve the nutritional value of these by-products, allowing its use for animal feed.[24] In addition to producing enzymes such as lipases, the fungus growth may increase protein content of the residues; it may increase the biodegradability facilitating the digestibility by ruminant animals; and it may reduce the content of polyphenols, which can affect the palatability of the residues.[25] In this way, all fractions after SSF can be exploited by agro-food industries.

The aim of this work was to valorize OP by SSF to produce lipase using a traditional tray-type bioreactor and a novel pressurized bioreactor. Different aspects were evaluated such as the need for sterilization, moisture content (MC) control, the substrate bed height and the air pressure and aeration rate. In addition, the fermented substrate was characterized to evaluate its potential applicability for animal feed.

MATERIAL AND METHODS

Biological material and substrates

Aspergillus ibericus MUM 03.49 (MUM culture collection, Braga, Portugal) is a black *Aspergillus*, isolated from wine grapes.[26] The fungus was grown on malt extract agar (MEA) plates (20 g L^{-1} malt extract, 20 g L^{-1} glucose, 1 g L^{-1} peptone and 20 g L^{-1} agar) at 30 °C for 7 days and stored at 4 °C. Spore suspension of the inoculum was prepared by adding peptone solution (1 g L^{-1} and 0.1 g L^{-1} Tween 80) to plate cultures. The spore concentration of the suspension was adjusted to 10^7 spores mL^{-1}. OP samples were collected from a two-phase extraction system at an olive mill plant in Vila Real, Portugal, and stored at −20 °C. Wheat bran (WB) was purchased in a local supermarket. Substrates were used without any pre-treatment.

SSF of olive pomace with wheat bran

SSF in flasks
SSF experiments were performed using OP mixed with WB, in a ratio of 1:1 (w/w, dry basis), 0.0133 g of (NH$_4$)$_2$SO$_4$ per gram of dry solid substrate, 33.33 μL g^{-1} of inoculum solution with 10^7 spores mL^{-1}.[27] The mixture of OP with WB resulted in optimum MC, of around 60% (w/w), without the need for its adjustment. Fermentations were carried out at 30 °C in an incubator for 7 days without agitation.[4,27] SSFs were performed using sterilized (121 °C, 200 kPa for 15 min) and unsterilized substrate.

SSFs in cotton-plugged 500 mL Erlenmeyer flasks were performed using 30 g of dry solid substrate. SSFs were performed in triplicate, for experiments with and without sterilization. To visually compare the microorganism growth, a SSF control experiment using unsterilized and non-inoculated substrate was performed.

SSF in tray-type bioreactor
The tray-type bioreactor used consisted of an incubator (112 × 48 × 45 cm) (FOC 225E Velp Scientifica, Usmate Velate MB, Italia) containing four (non-perforated) stainless steel trays (38 × 26 × 5 cm). SSFs were performed using sterilized and unsterilized substrate, in quadruplicate. Substrate was sterilized in covered glasses and the incubator and trays were sterilized with 76% (v/v) ethanol, before inoculation. Each tray was filled with 300 g of dry solid substrate and inoculated, resulting in a substrate bed height of 2.5 cm. In SSF with MC control, MC was adjusted by adding sterile distilled water, once a day.

Another SSF was performed using 500 g of dry solid substrate in (non-perforated) smaller trays (20 × 25 × 10 cm), and covered with a polymeric (polyethylene) film, to avoid MC reduction. The substrate was not sterilized, and it was inoculated. A substrate bed height of 8 cm was obtained. Temperature of the substrate bed was monitored in all experiments.

SSF in pressurized bioreactor
SSFs were carried out in a 19 L (42 cm height and 24 cm diameter) pressurized bioreactor (4555, Parr Instrument Company, USA). SSFs were performed using 500 g of dry solid unsterilized substrate, with bed height of 8 cm. Continuous absolute air pressures of 200 kPa, 400 kPa and 700 kPa were selected by the inlet air pressure setting and by controlling the regulatory inlet and outlet air valves. Air was continuously sparged in the substrate bed at the bottom of the bioreactor, at aeration rates of 1 L min^{-1} and 2 L min^{-1}, measured at the outlet gas conditions. Fermentation temperature was performed at 30 °C, using a thermostatic bath connected to a serpentine inside the pressurized bioreactor. SSF experiments were performed in duplicate.

Enzyme extraction after SSF
Fermented substrate was mixed with extracting solution composed of 10 g L^{-1} Triton X-100 in the proportion 5 mL per gram of dry solid substrate and incubated at 170 rpm and 20 °C for 2 h. The whole fermented substrate from flasks and pressurized bioreactor were used for the extraction; in the tray-type bioreactor the substrate was divided into six equal parts and each part was used as a

Table 1. Characteristics of olive pomace (OP), wheat bran (WB) and fermented substrate (SSF OP+WB). Values are the mean of triplicate analyses ± standard deviation (SD)

Characteristic	Value ± SD			
	OP	WB	OP + WB	SSF (OP + WB)
MC (%, w/w)	73.5 ± 0.4	12.5 ± 0.1	58.2 ± 0.3	65 ± 2
Total solids (%, w/w)	26.5 ± 0.4	87.5 ± 0.1	41.8 ± 0.3	35 ± 2
Ash (g kg^{-1})	66 ± 5	62.4 ± 0.2	64 ± 3	22.6 ± 0.8
Lignin (g kg^{-1})	432 ± 5	29.7 ± 0.5	231 ± 3	332 ± 11
Hemicellulose (g kg^{-1})	223 ± 8	285 ± 8	254 ± 4	232 ± 1
Cellulose (g kg^{-1})	125 ± 9	290 ± 3	208 ± 6	160 ± 4
Lipids (g kg^{-1})	167 ± 1	36.6 ± 0.4	102 ± 1	24 ± 3
Protein (g kg^{-1})	4 ± 1	8.6 ± 0.2	6 ± 1	1.5 ± 0.3
Reducing sugars (g kg^{-1})	96 ± 6	15 ± 2	56 ± 4	17 ± 2
Phenols in water extract (g kg^{-1})	8.4 ± 0.3	3.8 ± 0.3	6.1 ± 0.3	4.9 ± 0.7
Phenols in MeOH extract (g kg^{-1})	11 ± 0.9	5 ± 0.1	8 ± 0.2	5.8 ± 0.6
Antioxidant activity (mmol Trolox kg^{-1})	55 ± 0.3	5.6 ± 0.7	30.6 ± 1.9	10.3 ± 0.3
Antioxidant activity (IC$_{50}$)	1.25	18.2	2.6	8.1
N (%, w/w)	0.6 ± 0.1	2.57 ± 0.04	1.6 ± 0.1	2.87 ± 0.04
C (%, w/w)	49.7 ± 0.7	44.3 ± 0.2	47 ± 0.5	49.0 ± 0.2
C/N ratio	83 ± 7	17 ± 5	25 ± 6	17 ± 5
Crude protein (%, w/w)	3.8 ± 0.6	16.1 ± 0.3	9.9 ± 0.4	17.9 ± 0.3
Ca (g kg^{-1})	1.16 ± 0.04	1.85 ± 0.09	1.51 ± 0.07	2.3 ± 0.5
K (g kg^{-1})	17 ± 1	18 ± 1	18 ± 1	5.8 ± 0.5
Mg (g kg^{-1})	0.474 ± 0.22	5.1 ± 0.3	2.8 ± 0.3	2.9 ± 0.4
Zn (mg kg^{-1})	12 ± 0	145 ± 3	79 ± 2	96 ± 21
Cu (mg kg^{-1})	11.5 ± 0.7	14 ± 0	12.8 ± 0.4	17 ± 4
Fe (mg kg^{-1})	42 ± 2	134 ± 4	88 ± 3	103 ± 10
Mn (mg kg^{-1})	8.6 ± 0.1	128.5 ± 0.7	68.6 ± 0.4	74 ± 13
Cr (mg kg^{-1})	<22	<15	<19	<15
Ni (mg kg^{-1})	<22	<15	<19	<15
Pb (mg kg^{-1})	<22	<15	<19	<15
Na (mg kg^{-1})	373 ± 35	96 ± 1	235 ± 18	116 ± 17

sample. Enzymatic extracts were then centrifuged ($12000 \times g$ and 10 min at 4 °C) and filtered. The resulting enzymatic extracts were immediately used for lipase determination.

Analyses of enzymes and protein

Lipase activity was determined by a spectrophotometric method, using *p*-nitrophenyl butyrate in potassium phosphate 50 mmol L^{-1} at pH 7.0 and 37 °C for 15 min. The absorbance was measured at 405 nm, as described by Oliveira *et al.*[28] 1 unit of lipase activity (U) was expressed as the amount of enzyme that produces 1 μmol of *p*-nitrophenol per min, under the assay conditions. Lipase activity was expressed as units per gram of dry solid substrate (U g^{-1}).

The protein content of the extract was determined by Bradford's method, using bovine serum albumin (BSA) as standard.[29] Protein concentration was expressed as gram of protein per kilogram of dry solid substrate (g kg^{-1}). Specific activity was obtained by the ratio between lipase activity and protein concentration. It was expressed as units of lipase activity per milligram of total protein (U mg^{-1}). All analyses were performed in triplicate.

Other enzymes were determined in the enzymatic extract after 7 days of SSF using sterilized substrate. Xylanase, cellulase and β-glucosidase were determined through the methodology described by Salgado *et al.*[25] Lignin peroxidase, manganese peroxidase and laccase were determined through the methodology described by Salgado *et al.*[30] Enzyme activity was expressed as units per gram of dry solid substrate (U g^{-1}).

Characterization of substrates before and after SSF

The composition of substrates before and after SSF was determined to evaluate the increase of nutritional quality and potential applicability for animal feed. Initial characterization of OP was performed by Leite *et al.*[31] and is presented in Table 1. Characteristics of WB and of fermented substrate (SSF OP + WB), such as MC, total solids, ash, lignin, hemicellulose, cellulose, lipids, protein concentration, reducing sugars, phenols, nitrogen, carbon, crude protein, minerals and heavy metals, were determined according to Leite *et al.*[31] and Salgado *et al.*[25]

The extracts for antioxidant determination in unfermented and fermented substrates were obtained by methanol extraction with liquid:solid ratio of 20:1 (v/w). The extraction was carried out in Pyrex bottles in an incubator shaker at 50 °C, 100 rpm and 1 h, and the extracts were filtered. Antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay described by Dulf *et al.*[32] Known amounts of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were used for calibration. The free radical scavenging activity of the extracts was expressed as millimoles of Trolox equivalent per kilogram of dry solid substrate (mmol kg^{-1}), and as concentration of sample that reduces the DPPH radical scavenging activity at 50% (IC$_{50}$).

Since the ratio (dry basis) used in SSF was 1:1 (w/w), an average between the initial characteristics of OP and WB was determined, to observe modifications in the concentration of compounds after

SSF. A characteristic increase (or reduction) in relation to the initial value was determined by the following equation:

$$XIR = \left(X_f - X_i\right)/X_i \times 100 \qquad (1)$$

where *XIR* is a characteristic increase (or reduction) expressed in percentage (%), X_i is the initial value of a characteristic (before SSF), and X_f is the final value of a characteristic (after 7 days of SSF).

Analysis of experimental data

The data obtained were analyzed statistically using SPSS (IBM SPSS Statistics, Version 22.0. Armonk, NY: IBM Corp.) to study the effect of variables on lipase production and its specific activity. Data were tested for homogeneity, submitted to one-way analysis of variance (ANOVA) and a pair-wise multiple comparison procedure (Tukey test), at a confidence level of 95%.

RESULTS AND DISCUSSION

Effect of sterilization on lipase production by SSF in flasks

The evaluation of sterilization effect on lipase production was first carried out in Erlenmeyer flasks using sterilized and unsterilized inoculated substrates. A SSF control experiment was performed with unsterilized and non-inoculated substrate. The growth of *A. ibericus* was visually observed in both inoculated substrates. In the control assay, the growth of different autochthonous microorganisms producing different green and brown colonies was observed, unlike the black spots observed in flasks inoculated with *A. ibericus*. In this sense, the inoculation with *A. ibericus* allowed fast growth and avoidance of the proliferation of autochthonous microorganisms. In the olive ecosystems, different microorganisms can be found, such as yeasts,[33] bacteria[34] and filamentous fungi.[35] In particular, Baffi *et al.*[35] isolated 14 species of fungi from olive ecosystems, belonging to seven different genera (*Aspergillus*, *Penicillium*, *Rhizomucor*, *Mucor*, *Rhizopus*, *Lichtheimia* and *Galactomyces*). However, only sterilization of the substrate can remove completely the wild microbial population, contributing to better colonization with *Aspergillus* strain used as inoculum.

Lipase production was higher in SSF using sterilized substrate (Fig. 1). In flasks using unsterilized substrate, lipase production and specific activity decreased by 34% and 40%, respectively. On the other hand, in control experiment (unsterilized and non-inoculated) a lower lipase production was achieved. The sterilization and inoculation with *A. ibericus* spores on OP and WB mixture led to 2.7-fold increase in lipase production, compared with the control fermentation.

The positive effect of substrate sterilization in small-scale fermentations may be also due to the modification of the solid structure, which leads to the formation of micropores in the substrate, incrementing the specific surface area,[36] and improving the water solubility and water binding properties.[37] Consequently, the accessibility of the fungus and its enzymes to the micro and macronutrients of the substrate (OP + WB) may increase. For these reasons, sterilization step may be considered as a pre-treatment of substrate. In this sense, Pensupa *et al.*[38] observed that the production of cellulases with substrate treated by autoclave and dilute acid was similar and improved the results compared with untreated substrate.
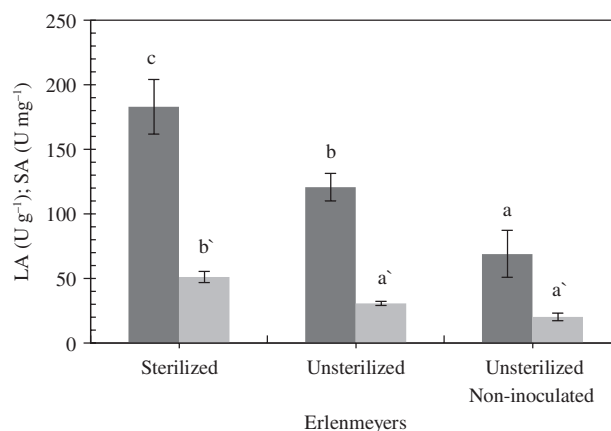

Figure 1. Results of (■) lipase activity (LA) and (■) specific activity (SA) of SSF in 500 mL Erlenmeyer flasks, using sterilized and unsterilized substrate, and using unsterilized and non-inoculated substrate. Depicted values are the mean of three independent fermentation experiments ± standard deviation. Means with the same letter do not differ significantly at $P > 0.05$ (Tukey test).

Table 2. Results of lipase activity (LA), specific activity (SA) and moisture content (MC), from SSF in a tray-type bioreactor, with and without MC control, and using unsterilized and sterilized substrate. Values are the mean of four trays ± standard deviation (SD). Means with the same letter do not differ significantly at $P > 0.05$ (Tukey test)

SSF conditions	LA ± SD (U g^{-1})	SA ± SD (U mg^{-1})	MC ± SD (%, w/w)
Without MC control, sterilized	74 ± 10^a	22 ± 2^a	27 ± 3^a
With MC control:			
Sterilized substrate	111 ± 14^b	36 ± 6^b	66 ± 3^b
Unsterilized substrate	131 ± 16^b	25 ± 4^a	67 ± 3^b

SSF in tray-type bioreactor

SSF was performed in a tray-type bioreactor using 300 g of dry solid sterilized substrate, with and without MC control. In SSF without MC control, MC loss rate of the trays was higher in the first days of fermentation and decreased with time. After 7 days of fermentation (Table 2), lower lipase activity was obtained, since MC decreased by 55% (from 60% to 27%). However, in SSF with MC control, higher lipase production was obtained, leading to 50% increase of lipase activity and 63% increase of specific activity. MC control minimizes the negative effects of low MC, such as the reduction in the solubility of nutrients of the substrate.[39]

The 10-fold scale increase of SSF from flasks to tray-type bioreactor with MC control in sterile conditions led to a significant decrease (40%) in lipase production and 29% decrease of specific activity. This decrease could be caused by the slight dehydration of the substrate (an average of 10% MC loss) during each day of SSF. Also, the accumulated heat inside the substrate bed, produced by fungus growth and metabolic activity, increases as the load substrate and/or bed height increases. This can lead to temperature increase and consequently to MC loss. In fact, the temperature in the tray-type bioreactor over the fermentation time increased from 30 °C to 34.3 °C, after 1 day of fermentation. The increase of temperature above optimum level (30 °C) for lipase production by *A. ibericus* has a negative impact on final activity.[4]
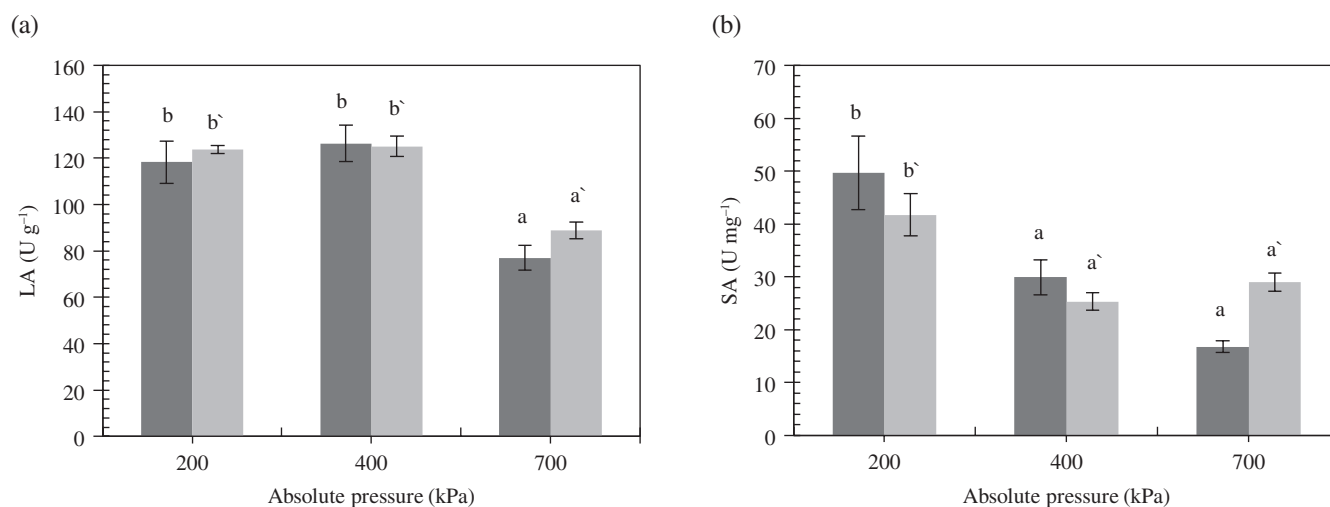
(a)

(b)

Figure 2. Results of (a) lipase activity (LA) and (b) specific activity (SA) of SSF of unsterilized substrate in a pressurized bioreactor at different absolute air pressures and at (■) 1 L min⁻¹ and (■) 2 L min⁻¹ of aeration rate. Depicted values are the mean of two independent fermentation experiments ± standard deviation. Means with the same letter do not differ significantly at $P > 0.05$ (Tukey test).

In general, the increase of substrate bed height has significant negative impact in lipase production in a tray-type bioreactor. Edwinoliver *et al.*[40] scaled-up the lipase production from flasks with 10 g of substrate to tray-type bioreactor with 100 g (0.8 cm substrate bed height) and 1 kg (1.5 cm substrate bed height), and reported a reduction of 4% and 17%, respectively, in lipase produced by *A. niger* on WB, coconut oil cake and raw wheat. The decrease of lipase activity was attributed to the temperature increase during the culture growth. Vaseghi *et al.*[9] reported an optimum substrate bed height of 0.5 cm, in comparison with using 3 cm, for SSF on sugarcane bagasse for *Rhizopus oryzae* lipase production in a tray-type bioreactor, yielding around 200 U g⁻¹. The substrate bed height may be reduced by increasing the tray area, to prevent temperature gradients and maximizing lipase production.[10]

In SSF experiments with MC control using 300 g of substrate, no significant impact of substrate sterilization was observed (Table 2). Thus, at larger scale, the SSF process is feasible using substrate without pre-treatment, as autoclave sterilization, where the fungus *A. ibericus* can grow and produce lipase.

SSF at pressurized bioreactor

SSF process in a pressurized bioreactor was carried out with 500 g of unsterilized substrate. Figure 2 presents the results of SSF in the pressurized bioreactor, at aeration rates of 1 L min⁻¹ and 2 L min⁻¹, and at different absolute air pressures. Maximum lipase activity (126 ± 8 U g⁻¹ at 1 L min⁻¹ and 400 kPa) was found at lower air pressures of 200 kPa and 400 kPa, without statistical difference between these results. However, maximum specific activity was obtained at 200 kPa. Results of lipase and specific activities were similar at aeration rates of 1 L min⁻¹ or 2 L min⁻¹. Also, no significant differences in MC of fermented substrate at different conditions of air pressure and aeration rate were observed. Similarly, Oliveira *et al.*[27] found equal lipase production by SSF in a packed-bed bioreactor, using 25 g of substrate, at aeration rates of 0.05 and 0.1 L min⁻¹.

The results of lipase activity obtained in the pressurized bioreactor with unsterilized substrate, under 200 kPa or 400 kPa and 1 L min⁻¹ or 2 L min⁻¹, were statistically similar to the lipase obtained in flasks with unsterilized substrate. However, about 60% significant increase was observed in specific activity, indicating that the pressure increase may be an important operating factor for optimization of enzyme production, since it may affect differently the expression of several enzymes.

Another SSF was performed in a tray-type bioreactor, using the same amount of substrate (500 g) and bed height of 8 cm, to compare the results obtained in a pressurized bioreactor with that in a tray-type. Trays were covered with a perforated polymeric film to simulate the close environment as in the pressurized bioreactor. The coverage of the tray prevented MC loss. Lipase production in the tray-type using 500 g substrate yielded 61 ± 13 U g⁻¹, significantly lower (around 50%) than all the values obtained under other conditions tested. This result may be attributed to the temperature rise inside the substrate bed that increased dramatically after 1 day of fermentation to around 36 °C. Moreover, mycelium growth was observed in all the substrate of the trays, but sporulation occurred only at the substrate surface, indicating oxygen limitations through the substrate bed.

Contrarily, in the pressurized bioreactor, fungus growth and sporulation were observed in all substrates, at all conditions tested. In aerobic cultures, total air pressure is a key controlling factor, as it alters the dissolved gas partial pressure, in particular, the dissolved oxygen, which may be a limiting factor for cell growth and metabolic activity.[17] The effect of pressure on the microorganism metabolism depends on the culture conditions, pressurization mode and the microorganism itself.[17] The continuous pressurized aeration in the bioreactor may allow efficient gas exchange over the fermentation time and the dissipation of excessive heat derived from fungus growth, even using a high substrate bed height. This proved that the use of a pressurized bioreactor may effectively maintain lipase production with high substrate bed height.

SSF under increased air pressure has been well explored and there is some work using air pressure pulsation instead of continuous air pressure, as was used in this work. Aijun *et al.*[20] applied air pressure pulsation SSF, and found that the protease of *Bacillus pumilus* increased 63% and 95%, in relation to static SSF, when pressure amplitude used was 50 kPa and 100 kPa, respectively. This process supplied sufficient oxygen and enlarged the aerobic
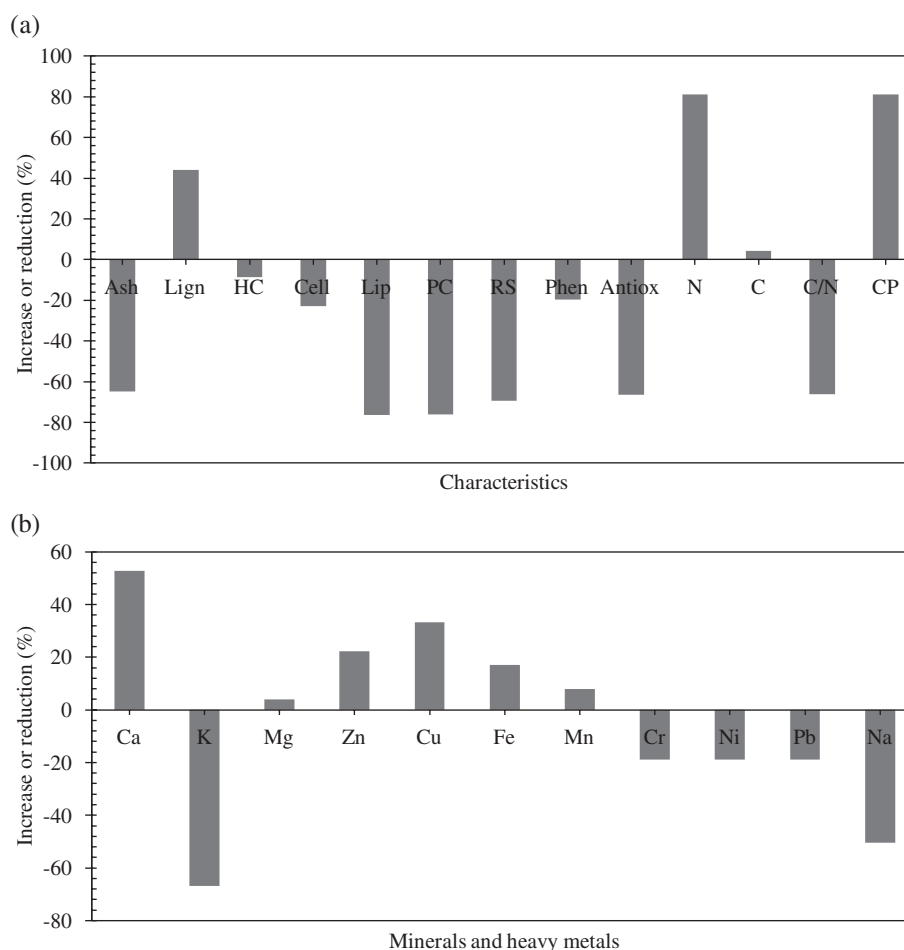
(a)

(b)

Figure 3. Increase (positive values) or reduction (negative values) of (a) the characteristics and (b) minerals and heavy metals of the fermented substrate, after 7 days of SSF using sterilized substrate.

surface for microorganism growth.[20] Similarly, Zeng and Chen[21] applied cycles of 20 min without pressure increase and 10 s pulses of air at 200 kPa in SSF, and observed that feruloyl esterase production by *A. niger* improved and the fermentation time to achieve the highest enzyme production was shortened, compared with static SSF.

This work demonstrated that SSF under pressurized air could improve specific activity, being favorable to the enzyme purification step. However, when using higher pressures (700 kPa) the lipase activity decreased, probably due to unbalanced oxidative stress. To our knowledge, this is the first report on the study of *A. ibericus* lipase production by an SSF process under continuous pressurized aeration conditions.

Modifications in substrate composition after SSF

The substrates (OP and WB) were analyzed before and after 7 days of SSF, to observe the nutritional composition modification. Table 1 presents the main chemical parameters of these substrates. Figure 3 presents the respective increase or reduction of the characteristics after SSF, in relation to the initial characteristic value of the substrate (mixture 1:1 of OP with WB).

The crude protein is an important parameter to consider in animal feed. Through SSF, the crude protein content increased by 81%, reaching 18% (w/w) in the dry solid fermented substrate. This increase is correlated with the biomass produced from fungal

growth during SSF. The protein content in similar species to *A. niger* is estimated at 27% (w/w).[41] Results confirmed the efficacy of SSF to enhance the protein content of the fermented substrate. Moreover, the value of crude protein achieved in the fermented solid is within the range of protein used in animal feed.[25] For example, basal broiler chicken diet includes a crude protein level of 18.5% during the first 7–8 weeks and 16% for the subsequent 9–14 weeks.[42] Similarly to this work, Li *et al.*[42] obtained a crude protein increase of 80%, using *A. niger* on SSF of solid wastes from *Silybum marianum* fruits. Salgado *et al.*[25] also observed an increase of 107% using *A. uvarum* by SSF using mixture of winery by-products and OP. Canedo *et al.*[24] found a 100% increase in crude protein content after 7 days of SSF in the fermented wastes of brewery spent grain using *Rhizopus oligosporus*. The use of *A. ibericus* in SSF is safe as mycotoxins are not produced,[26] it provides a protein concentrate, and may give an alternative use of the by-products from the olive mill industry as animal feed. After SSF, reductions of 9% and 23% in hemicellulose and cellulose, were observed (Fig. 3), respectively. Lignocellulolytic enzymes such as cellulases and xylanases, and the enzyme β-glucosidase were detected in the extracts at activities of $5.1 \pm 0.6 \, U \, g^{-1}$, $9.1 \pm 0.5 \, U \, g^{-1}$ and $48 \pm 1 \, U \, g^{-1}$, respectively. These enzymes caused slight degradation of hemicellulose and cellulose. These reductions may improve the digestibility and accessibility of these components in the digestive tract of animals.[25] On the other hand, lignin was not consumed and the presence of ligninases as lignin peroxidase,

laccase and manganese peroxidase, was not detected. Thus, the lignin relative proportion and concentration increased in the fermented substrate, compared with the unfermented one.

Phenolic compounds were reduced by 20% after 7 days of SSF by *A. ibericus*, even without the presence of laccases and peroxidases. Filamentous fungi are able to degrade phenolic compounds.[43] In previous work, the same strain of *A. ibericus* was used in SmF of diluted olive mill wastewater, where reductions of 28% and 37% in phenolic compounds were observed.[5] Similarly, Salgado *et al.*[25] observed a reduction of 28% in phenolic compounds by *A. uvarum* after 10 days of SSF, using a mixture of winery by-products and OP. Reduction of phenolic compounds in the fermented substrate was positive, since they negatively affect the animal's feed intake, feed digestibility and production efficiency.[25]

The antioxidant activity of unfermented and fermented substrates was determined (Table 1). High antioxidant activity of extracts from OP and low antioxidant activity of extracts from WB before SSF were observed. After SSF, a reduction in antioxidant activity in fermented substrate was observed, due to the extraction of phenolic compounds during enzyme recovery. The antioxidant activity in enzymatic extracts was 14.2 ± 1 mmol Trolox kg^{-1}; the sum of antioxidant activity in extract from fermented substrate and enzymatic extract was 24.5 mmol Trolox kg^{-1}. Thus, the antioxidant capacity was reduced after SSF. This may have been due to the reduction of phenolic compounds by fungus, as can be seen in methanolic extracts before and after SSF (Table 1). However, the antioxidant activity in fermented substrate maintained an interesting level, which was similar to the values of antioxidant activity in 112 medicinal plants.[44]

Lipids suffered a drastic reduction of 76%, after 7 days of SSF. This was a result of SSF optimization for lipase production. A final lipids concentration of $2.4 \pm 0.3\%$ was found. The concentration obtained is interesting for animal feed, since the lipids found in grains and forages naturally make up approximately 2–4% of the feed.[45]

Figure 3 presents the modifications in the percentage of minerals and heavy metals of the fermented substrate, in relation to the unfermented one. Highest reductions were found for potassium and sodium contents, and highest increases for calcium and copper. Results are in agreement with the literature for copper, zinc, manganese and iron increases.[25,46] Joshi and Sandhu[46] reported increases in those minerals after SSF of WB using different yeast species, *Saccharomyces*, *Candida* and *Torula*. Also, Salgado *et al.*[25] observed an increase of iron in the substrate by SSF of a mixture of winery by-products and OP using *A. uvarum*. The reduction of sodium was confirmed by Joshi and Sandhu[46] and Salgado *et al.*[25] Contrary to the results presented, these authors found an increase in potassium and reduction in calcium and magnesium.[25,46] An increase in microelements (calcium, magnesium, zinc, copper, iron and manganese) by fermentation improved the nutritive value of OP with WB, with possible higher availability to livestock. In addition, reductions of heavy metals contents were observed (chromium, nickel and lead).

CONCLUSIONS

Sterilization of the substrate for SSF at small scale led to higher lipase production. However, it was proved that at large scale, the sterilization step is not necessary. SSF was successfully performed in a tray-type bioreactor but MC loss needs to be prevented. For the first time, SSF was successfully performed in a pressurized bioreactor for lipase production. SSF in this bioreactor seemed to be more effective than in the tray-type, improving specific lipase activity. This work presented an integrated waste valorization process using OP residue for lipase production and nutritional value increase of the fermented substrate.

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