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RESEARCH PAPER

Optimization of lipase production by solid-state fermentation of olive pomace: from flask to laboratory-scale packed-bed bioreactor

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Abstract Lipases are versatile catalysts with many applications and can be produced by solid-state fermentation (SSF) using agro-industrial wastes. The aim of this work was to maximize the production of *Aspergillus ibericus* lipase under SSF of olive pomace (OP) and wheat bran (WB), evaluating the effect on lipase production of C/N ratio, lipids, phenols, content of sugars of substrates and nitrogen source addition. Moreover, the implementation of the SSF process in a packed-bed bioreactor and the improvement of lipase extraction conditions were assessed. Low C/N ratios and high content of lipids led to maximum lipase production. Optimum SSF conditions were achieved with a C/N mass ratio of 25.2 and 10.2% (w/w) lipids in substrate, by the mixture of OP:WB (1:1) and supplemented with 1.33% (w/w) $(NH_4)_2SO_4$. Studies in a packed-bed bioreactor showed that the lower aeration rates tested prevented substrate dehydration, improving lipase production. In this work, the important role of Triton X-100 on lipase extraction from the fermented solid substrate has been shown. A final lipase activity of 223 ± 5 U g^{-1} (dry basis) was obtained after 7 days of fermentation.

Keywords Olive pomace · Solid-state fermentation · *A. ibericus* · Lipase production · Packed-bed bioreactor

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Introduction

The interest in the production of lipases is associated with their applications as additives in the food industry, fine chemicals, detergents, wastewater treatment, cosmetics, pharmaceuticals, leather processing and biomedical assays [1]. The global market of enzymes has grown in recent years. In 2011, its value was about $4 billion and it is estimated to achieve $6 billion in 2016 [2]. The market of lipase is projected to reach $590.5 million by 2020 [3]. Following proteases and carbohydrases, lipases are considered the third largest group, based on total sales volume [4]. Many enzyme manufacturers use submerged fermentation (SmF) techniques for their production [5]. However, in recent years, solid-state fermentation (SSF) has been shown to be very promising for enzymes and other bioproducts production at very interesting concentrations [6].

SSF is a fermentation process which involves the culture of microorganisms on moist solid supports [7]. SSF simulates the natural habitat of fungi and is, therefore, the preferred choice for these microorganisms to grow and produce useful value-added products [8]. SSF offers numerous opportunities in processing of agro-industrial residues that can be used as solid supports [7]. In addition, SSF is environmentally friendly since it produces less wastewater and has low energy requirements and the products are obtained at higher concentrations than in SmF with important cost saving in downstream processing operations [8].

The selection of suitable solid substrate composition is a key factor in SSF. Several aspects should be taken into

account such as the source of C and N, inducers of enzyme expression, costs and availability of substrates, particle size and moisture content (MC) [6]. The source of nitrogen and the C/N ratio are important parameters of SSF which should be optimized to maximize biomass and enzyme production [9], and thus minimize the cost of the process. Several works have demonstrated that the addition of nitrogen to agro-industrial wastes can improve enzyme production [10–12]. The evaluation of nitrogen sources (organic and inorganic) is important, because their use is subject to metabolic versatility, as well as economic implications [13]. The C/N ratio of agro-industrial wastes can indicate the need to supplement the substrate with a source of N. Usually the optimum value of C/N for enzymes production is between 10 and 20 [13].

Olive mill wastes are agro-industrial wastes that are obtained from olive oil extraction and represent an environmental problem in Mediterranean countries [14]. Olive oil is produced mainly in Mediterranean countries, representing 93% of the total production in the world, with a production of around 3 billons tons of olive oil in 2014 [15]. Spain is the major virgin olive oil producer, where, in 2014, 1.7 billons tons were produced, followed by Italy (around 300,000 tons), Greece (210,000 tons), Tunisia (180,000 tons), Morocco (140,000 tons) and Portugal (66,500 tons) [15]. Olive mill wastes are produced in large quantities in short periods of time and must be properly disposed to avoid environmental risks [14]. Olive pomace (OP) is the main waste generated in the extraction of olive oil by the two-phase extraction system. The main OP disposal option consists of a second extraction of the remaining pomace oil prior to combustion, but this approach has environmental impact [16]. The three-phase system is widely used in Italy, Greece and other Mediterranean countries [17]. However, in Portugal and Spain the two-phase extraction system was largely implemented in the olive mills, turning OP (solid fraction) to be the most important by-product produced. In Portugal, 84% of olive oil is extracted by two-phase system, producing more than 350 thousand tons of OP, during the year 2015 [18].

OP is an acidic and very humid material, rich in organic matter, minerals and fats [19], which can be valorized for biotechnological applications. The applications reported in literature include composting [20]; production of hydrogen, methane and ethanol as biofuel [21, 22]; production of polymers [23] and enzymes, such as lignocellulosic ones [24]. Also, OP can be used for lipase production due to the residual oil content [12, 25]. However, OP is characterized by a low N content [26, 27]; thus their C/N ratio is not suitable for SSF and should be corrected by the addition of a N source and/or a mixture of different solid substrates [1]. Wheat bran (WB) is a substrate which can be used due to its high content in carbohydrates and proteins [28]. Some authors have proposed the combination of WB with an oil cake (agro-industrial residue from oil extraction) for the improvement of lipase production [29–31].

The evaluation of laboratory-scale bioreactor is a necessary step before carrying out the industrial scale-up. A packed-bed bioreactor is one of the bioreactor types suitable for SSF. It typically involves a static bed on top of a perforated plate through which conditioned air is blown. These bioreactors have several advantages, such as they are easy to handle and to operate continuously, and allow the extraction of enzymes in situ [32].

Thus, with the aim of improving the production of lipase by *Aspergillus ibericus* MUM 03.49 using SSF of OP with WB, several variables affecting lipase secretion were studied, such as source of nitrogen and composition of mixtures of OP and WB. Under the best conditions obtained, the process was implemented in a packed-bed bioreactor and the effect of aeration rate was assessed. Additionally, lipase extraction conditions were optimized.

Materials and methods

Microorganism and substrates

Aspergillus ibericus MUM 03.49 (MUM culture collection, Braga, Portugal) was used. 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 suspensions of the inoculum were prepared by adding peptone solution (1 g L^{-1} peptone 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 olive mill plant in Vila Real, Portugal, and stored at −20 °C. WB was purchased in a local supermarket. The characteristics of OP were determined by Leite et al. [27]. WB characteristics were determined through the methodology described by Leite et al. [27].

Optimization of lipase production by SSF

SSFs were performed in cotton-plugged 500 mL Erlenmeyer flasks containing 30 g dry solid substrate in a ratio of 1:1 (w/w, dry basis) of wet OP and WB [25]. The mixture of OP with WB resulted in a MC of around 60%, without the need of adjustment. Flasks were prepared, autoclaved at 121 °C for 15 min, cooled, inoculated with 1 mL of inoculum suspension and incubated at 30 °C for 7 days. After the incubation period, the fermented substrates were extracted as described below to obtain the enzymatic extracts.

Different sets of SSF experiments were conducted to study the influence of the addition of different nitrogen sources and of substrate composition in the production of lipase by *A. ibericus*. Urea, $NaNO_3$, NH_4Cl and $(NH_4)_2SO_4$ were used as nitrogen sources at a concentration of 2% (w/w) in the dry solid substrate. $(NH_4)_2SO_4$ was further evaluated at concentrations that ranged from 0 to 3.33%, since it was the nitrogen source with the most pronounced effect in the previous evaluation.

Different ratios of dry mixtures of OP and WB (OP:WB mass ratios of 1:0, 3:2, 1:1, 2:3, 1:4 and 0:1) and mixtures of olive oil [0, 1, 2.5, 5 and 10% (w/w)] with 30 g of dry solid WB were tested to evaluate the effect of different C/N ratios and content of lipids, phenols and reducing sugars on lipase production. In this set of experiments, MC was adjusted to 60% (w/w). Here, multiple linear regression analysis was used to describe the effect of these variables on lipase production.

After the optimization of SSF conditions, a set of experiments was performed to monitor the time course of lipase production. Flasks were prepared as described before and destructively sampled each 2 days over a period of 20 days.

SSF in a packed-bed bioreactor

The horizontal packed-bed bioreactor consisted of a double jacketed glass column (34 cm length and 3 cm internal diameter) connected to a filtered air supply. The air was passed through a 0.22 μm filter and bubbled in distilled water before entering the column. The air flow rate was measured and controlled by a flowmeter (Aalborg Instruments & Controls, Inc., USA). The bioreactor and 25 g of dry solid substrate were previously sterilized separately. The substrate was inoculated with 1 mL of inoculum suspension and mixed. The column was completely filled with the inoculated substrate and incubated at 30 °C for 7 days. SSFs were performed at different aeration rates of 0.05, 0.1 and 0.2 L min^{-1} and kept at 30 °C by circulating hot water, using a thermostatic bath. SSF experiments in the bioreactor were performed in triplicate. Also, an additional SSF without aeration was performed. After SSF, the fermented substrate was removed from the column and transferred to a 500 mL Erlenmeyer flask for enzyme extraction.

Extraction and lipase determination

The fermented substrates were mixed with 5 mL of a solution with 5 g L^{-1} Triton X-100 and 10 g L^{-1} NaCl per gram of dry solid substrate, and shaked at 170 rpm and 20 °C for 2 h. Enzymatic extracts were then centrifuged (12,000×*g* for 10 min at 4 °C) and filtered using Whatman No. 1 filter paper [25]. Lipase activity was determined by a spectrophotometric method, using a reaction mixture composed of 5 μL of enzymatic extract with 300 μL of 2 mM *p*-nitrophenyl butyrate in potassium phosphate 50 mM at pH 7.0. The absorbance was measured at 405 nm after reaction for 15 min at 37 °C. One unit of lipase activity (U) was expressed as the amount of enzyme which produces 1 μmol of *p*-nitrophenol per minute, under the assay conditions. Lipase activity obtained was expressed as units per gram of dry solid substrate (U g^{-1}).

Optimization of lipase extraction

To assess if the lipase extraction conditions described above could be further optimized, variables such as type and volume of extracting solvent were varied. Samples of 30 g of dry solid were treated with 150 mL (5 mL g^{-1}) of each type of solvent (distilled water, phosphate buffer, NaCl, Tween 80 and Triton X-100 at different concentrations). Then, using the best solvent, the volume of solvent was varied from 2.5 to 7.5 mL g^{-1}. Five consecutive extractions were performed in the same substrate sample to determine the extraction recovery.

Analysis of experimental data

The data obtained were statistically analyzed using SPSS (IBM SPSS Statistics, Version 22.0. Armonk, NY, USA: IBM Corp.). Data were tested for homogeneity, submitted to one-way analysis of variance (ANOVA) and a pairwise multiple comparison procedure (Tukey test) at a confidence level of 95%. The multiple linear regressions were carried out using Statgraphics Centurion XVI software (Statpoint Technologies, Inc. Warrenton, V).

Results and discussion

Optimization of lipase production by SSF

Characterization of residues OP and WB

Residues characterization, OP and WB are presented in Table 1. OP is very humid; it contains reducing sugars, phenols and presents a high C/N mass ratio, indicating the need of nitrogen supplementation. OP also contains a considerable concentration of lipids, which induces lipase production [27]. These results are in the range found in the literature [16]. WB presents low MC, low lipid concentration and higher N content than OP. These results are according to the literature [28]. The low C/N ratio found in WB contributes to a C/N ratio adjustment by the combination of both residues.

Table 1 Characteristics of olive pomace (OP) [27], and wheat bran (WB)

Characteristics	Value ± SD	
	OP	WB
MC (%, w/w)	73.5 ± 0.4	12.5 ± 0.1
Total solids (%, w/w)	26.5 ± 0.4	87.5 ± 0.1
Ash (%, w/w)	6.6 ± 0.5	6.24 ± 0.02
Lipids (%, w/w)	16.67 ± 0.1	3.66 ± 0.04
Phenols (%, w/w)	0.84 ± 0.03	0.38 ± 0.03
Reducing sugars (%, w/w)	9.6 ± 0.6	1.5 ± 0.2
N (%, w/w)	0.6 ± 0.1	2.57 ± 0.04
C (%, w/w)	49.7 ± 0.7	44.3 ± 0.2
C/N ratio	83 ± 7	17 ± 5

Values are the mean of triplicate analysis ± standard deviation (SD)

Effect of nitrogen sources

In a previous work, Oliveira et al. [25] observed a positive influence of the substrate supplementation with $NaNO_3$ as a nitrogen source on lipase production by *A. ibericus*. Here, the influence of different nitrogen sources on lipase production was studied. Thus, the effect of urea, an organic source, and $NaNO_3$, NH_4Cl and $(NH_4)_2SO_4$, inorganic sources, was studied. For all the nitrogen sources used, a significant positive effect ($p < 0.001$) on lipase production was obtained by nitrogen source addition (Table 2), compared to the SSF without nitrogen supplementation. The highest lipase activity was obtained using 2% (w/w) of ammonium salts, NH_4Cl and $(NH_4)_2SO_4$ with C/N ratio close to 23.

A positive effect of using an inorganic source of nitrogen was observed. The production of lipase was 1.4 times higher using an inorganic source of nitrogen, such as ammonium sulfate, than using an organic source, such as urea. A similar effect was observed in the production of lipase by *Bacillus coagulans* in SSF of melon wastes, where the addition of $(NH_4)_2SO_4$ increased enzyme production 1.6 times compared to urea addition, while other organic nitrogen sources had no effect [33]. Also, Lopes et al. [34] found that the use of $(NH_4)_2SO_4$ led to improve production of lipase by *Yarrowia lipolytica* in SSF of OP with WB when compared with the use of urea. The same positive effect of inorganic nitrogen was described by Lima et al. [35] in the production of lipase by *Penicillium aurantiogriseum* in SmF. Kamini et al. [36] did not observe any difference in the addition of organic or inorganic nitrogen source in the production of lipase by *A. niger*. In addition, the use of organic nitrogen source may difficult the purification step of the enzymes after fermentation [35]. Thus, the addition of inorganic nitrogen can reduce the cost of the process, simplifying the downstream process.

Figure 1 presents the lipase yields of experiments conducted with different concentrations of $(NH_4)_2SO_4$. The increase of $(NH_4)_2SO_4$ concentration above 2% did not improve lipase production, but the decrease below 0.67% had a negative impact. In the present work, 1.33% (w/w) of $(NH_4)_2SO_4$ was selected to perform SSF in the following experiments.

Effect of C/N ratio and lipids on lipase production

Mixtures of OP, WB and olive oil were studied to evaluate the effect of C/N ratio and content of lipids of substrate on lipase production. Table 3 shows the mixtures used, their N content, C/N ratio, content of lipids, phenols and reducing sugars in the mixture and the respective lipase activity achieved. As can be seen, low C/N ratios (22–25) led to high lipase activities. No lipase production was observed in SSF with a C/N ratio of 56.5, and C/N ratios below the value of 22 reduced the lipase production. Often, the agro-industrial wastes have a high C/N ratio due to their low content in N as OP. Thus, these wastes are supplemented with N source to carry out the biotechnology processes, which increased the costs of the process. This can be avoided using a mixture of wastes to adjust the C/N ratio and by optimization of this ratio so that the substrate is not needlessly oversupplemented. Jia et al. [37] observed that this parameter had significantly influenced lipase production by *Aspergillus* sp. in SmF. However, its effect in SSF with *Aspergillus* sp. was not studied.

Other works studied lower C/N ratios for the production of lipase by SSF using *Penicillium* sp. Rigo et al. [38] evaluated the range of C/N ratios of 2–10, obtaining an optimum value at 6.1 using a substrate with high N content such as soybean bran. Gombert et al. [39] studied the influence of the C/N ratio on lipase production by *Penicillium restrictum* and achieved 30.3 U g^{-1} using babassu oil cake with a C/N ratio of 14.1. In the present work high

Table 2 Lipase activity (LA) affected by the different nitrogen sources at a concentration of 2% (w/w)

N source supplementation	Without N source	Urea	$NaNO_3$	NH_4Cl	$(NH_4)_2SO_4$
C/N mass ratio	29.7	18.8	24.7	22.4	23.5
LA ± SD (U g^{-1})	89 ± 5^a	106 ± 7^b	115 ± 6^b	144 ± 5^c	151 ± 7^c

Values are the mean of triplicate analysis ± standard deviation (SD). Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test)

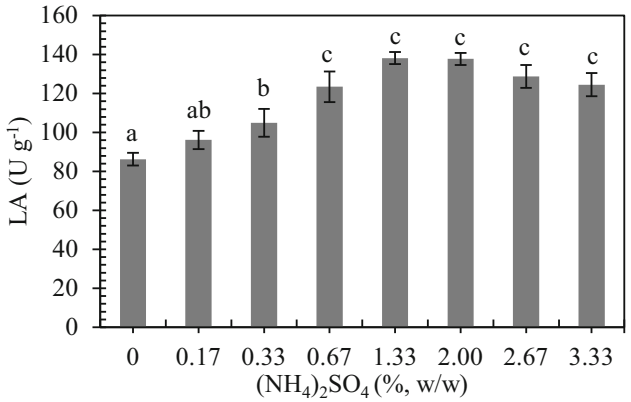

Fig. 1 Profile of lipase activity (LA) as a function of the amount of $(NH_4)_2SO_4$ added to the substrate. Depicted values are the mean of triplicate analysis ± standard deviation. Means with the *same letter* do not differ significantly at $p > 0.05$ (Tukey test)

enzyme activities were obtained at C/N ratio higher than 2–15. Thus, this gives the opportunity to use wastes with low N content.

The content of lipids may be one of the most important factors influencing the production of lipase in SSF. OP contains residual olive oil that acts as an inducer on lipase production. To evaluate the effect of lipids in lipase production, several mixtures of OP:WB and WB:olive oil were studied, which led to a wide range of lipids concentrations (3.66–16.7%). The maximum lipase production was achieved between 10.2 and 13.7% of lipids. As can be seen, the addition of 10% of olive oil to WB led to a lipase production of 152 ± 3 U g^{-1}, that is similar to the one obtained with the OP:WB in a ratio of 1:1. Other researchers also found that olive oil induces the production of lipase in SSF. For example, Palma et al. [40] found a positive effect of the addition of 1% olive oil to babassu cake for lipase production by *Penicillium restrictum*, achieving 17.2 U g^{-1}, a 1.7-fold increase compared to non-supplemented substrate. Also, Falony et al. [41] found a positive effect of adding 1.5% olive oil to WB, obtaining 9.1 U g^{-1} of lipase.

However, high concentrations of olive oil can limit oxygen transfer which could modify the microbial metabolism leading to less lipase production [42]. In this sense, Damaso et al. [43] observed a reduction of lipase production by *A. niger* with increasing olive oil addition to WB, and at 12% of olive oil lipase activity was no longer detected. The high content of lipids in OP could be one of the reasons for the low lipase activity in substrates with elevated OP:WB ratio (1:0 and 4:1). Thus, the mixture of OP with other agro-industrial wastes as WB can adjust the content of lipids to an optimum value for lipase production. These results showed the importance of the use of agro-industrial wastes containing residues of lipids as well as the mixture of them to improve lipase production.

The multiple linear regression analysis was used to describe the effect of these parameters on lipase production. In addition, other independent variables were evaluated such as the content of phenols and reducing sugars. These parameters can also affect the growth of fungus and the production of enzymes [27]. On the basis of statistical parameters shown in Table 4, the model Eq. (1) showed a good fit, since the value of the determination coefficient (R^2) was 0.9758. This value indicates that the model explains 97.58% of the variability in lipase production,

$$\mathrm{LA} = 158.24 - 8.54\ \mathrm{CNR} + 8.40\ \mathrm{LP} + 19.43\ \mathrm{RS}, \quad (1)$$

where LA is lipase activity (U g^{-1}), CNR is the C/N ratio, LP is the concentration of lipids (%, w/w), and RS is the concentration of reducing sugars (%, w/w). The content of phenols was not taken into account in the

Table 3 Results of experimental lipase activity (LA) from SSF performed with different ratios of olive pomace (OP) and wheat bran (WB) and from SSF performed with WB supplemented with different concentrations of olive oil; and respective variation on N content, C/N ratio and content of lipids, phenols and reducing sugars

OP:WB ratio	N (%, w/w)	C/N ratio	Olive oil addition (%, w/w)	Lipids (%, w/w)	Phenols (%, w/w)	Reducing sugars (%, w/w)	Experimental LA ± SD (U g^{-1})
1:0	0.88	56.5		16.7	0.84	9.6	3.6 ± 1.1
3:2	1.67	28.5		11.5	0.66	6.4	122 ± 2
1:1	1.87	25.2		10.2	0.61	5.6	144 ± 5
2:3	2.06	22.5		8.9	0.56	4.7	142 ± 2
1:4	2.46	18.5		6.3	0.47	3.1	109 ± 3
0:1	2.85	15.5	0	3.7	0.38	1.5	84 ± 6
0:1	2.86	15.8	1	4.7	0.38	1.5	93 ± 7
0:1	2.87	16.1	2.5	6.2	0.38	1.5	104 ± 4
0:1	2.88	16.7	5	8.7	0.38	1.5	112 ± 5
0:1	2.91	17.8	10	13.7	0.38	1.5	152 ± 3

Values of lipase activity are the mean of triplicate analysis ± standard deviation

Table 4 Analysis of variance for the regression model representing lipase activity

Source	SS	*df*	MS	*F* ratio	*p* value
Model	15,886.5	3	5295.52	80.55	0
Residual	394.452	6	65.742		
Total (corr.)	16,281	9			

$R^2 = 97.58\%$

R^2 (adjusted for *df*) = 96.37%

Standard error of est. = 8.11

Mean absolute error = 4.9

SS sum of squares, *df* degrees of freedom, *MS* mean squares

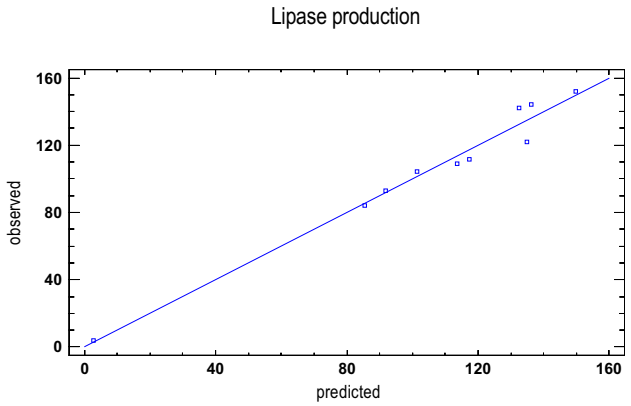

Fig. 2 Graphical representation of the observed and predicted values of lipase activity obtained when using the test data set

model since this variable had no statistical significant effect on LA. Figure 2 shows the experimental values versus model outputs for dependent variables, thus obtaining a heuristic evaluation of the performance. On the basis of these results, the lipase production by *A. ibericus* can be predicted by knowing C/N ratio and the content of lipids and sugars of the solid substrate. This is interesting, since OP composition varies in function of olive oil extraction conditions used [26].

Time course of lipase production

Figure 3 presents the results of lipase activity and its productivity over fermentation time. An increase in lipase production over time was observed, reaching 166 ± 5 and 209 ± 10 U g^{-1} after 10 and 20 days of fermentation, respectively. However, the maximum productivity was obtained on the 6th day (21 ± 1 U g^{-1} day^{-1}) with a lipase production of 127 ± 6 U g^{-1}. Using the same strain on OP at different SSF conditions, Salgado et al. [12] found the maximum lipase production on the 5th day, after which a stabilization over 12 days occurred. On the contrary,

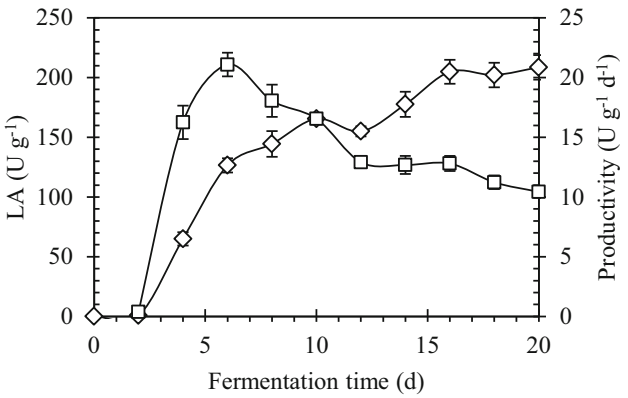

Fig. 3 Time course profiles of lipase activity (LA) (—◇—) and productivity (—□—) in SSF conducted at optimum conditions. Depicted values are the mean of triplicate analysis ± standard deviation

some authors observed maximum lipase production on the 3rd [44], 4th [30] and 5th days [45] of fermentation, respectively, with *P. simplicissimum* on babassu cake with sugarcane molasses, *A. niger* on wheat rawa, coconut oil cake and WB, and *A. niger* on WB. After the maximum, they also observed a decline with time.

SSF in a packed-bed bioreactor

Different aeration rates were evaluated in a packed-bed bioreactor for SSF. The results are presented in Fig. 4. The dehydration of the substrate was observed visually at the beginning of the column over fermentation time, even using saturated air. The highest values of lipase production were obtained at aeration rates of 0.05 and 0.1 L min^{-1}, using 25 g of substrate (2 and 4 mL min^{-1} g^{-1}, respectively), without statistically significant differences among the results at both conditions. An SSF without aeration was performed, where no fungal growth was observed and consequently lipase was not produced. The column was completely filled and maintained without aeration, thus limiting fungal growth due to the lack of oxygen. The aeration rate favors the transport of oxygen to the solid substrate. However, with the increase of aeration rate, lipase activity may decrease due to changes in fungal metabolism [46]. Reduction of lipase observed at 2 L min^{-1} could be attributed to excessive forced aeration used and by dehydration along the column derived from that. The results of lipase produced in a packed-bed bioreactor using 0.05 L min^{-1} were statistically similar to that ($p > 0.05$) obtained in flasks (144 ± 5 U g^{-1}—Table 3) after 7 days of SSF.

Pérez-Rodríguez et al. [47] found an optimum aeration rate of 0.1 L min^{-1} in a packed-bed bioreactor using 20 g

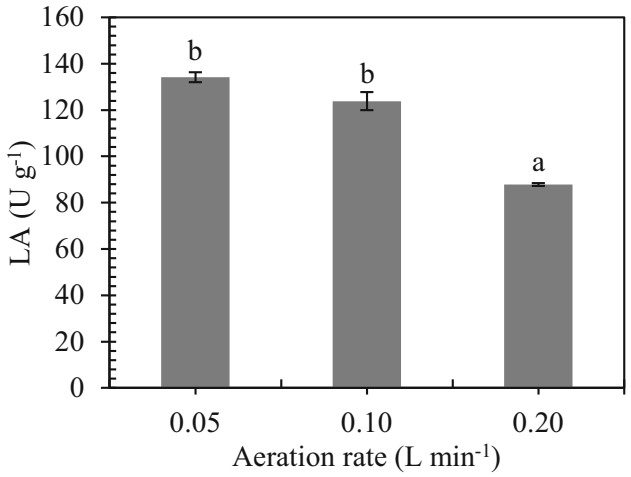

Fig. 4 Results of lipase activity (LA) of SSF in a packed-bed bioreactor at different aeration rates. Depicted values are the mean of three independent fermentation experiments ± standard deviation. Means with the *same letter* do not differ significantly at $p > 0.05$ (Tukey test)

corncob (5 mL min^{-1} g^{-1}) for xylanase production by SSF with *A. niger*. Using the same bioreactor, Salgado et al. [48] found an optimum aeration of 0.2 L min^{-1} using 20 g of mixtures of winery and olive mill wastes (10 mL min^{-1} g^{-1}) which improved cellulase and xylanase production by *A. uvarum*. In the production of citric acid using *A. niger*, Lu et al. [49] reported the need for an aeration rate of 1.5 L min^{-1} in a packed bed using 180 g of kumara (starch root crop) (8.3 mL min^{-1} g^{-1}) to maximize acid production.

Optimization of lipase extraction

To extract maximum lipase from the fermented solid substrates, the efficacy of the lipase extraction procedure was tested assessing the effect of the type of solvent and then the best volume of the solvent. The highest lipase activities were recovered when the fermented substrate was extracted with 7.5 mL of 10 g L^{-1} Triton X-100 per gram of dry solid substrate (Table 5). Thus, Triton X-100 aqueous solution appeared to be better and the role of NaCl was negligible. Triton X-100 is a non-ionic surfactant and it might solubilize the enzyme from the solid substrate to the emulsion, resulting in the highest recoveries of lipase. Similarly to these results, some authors also found 10 g L^{-1} Triton X-100 as the best solvent for lipase extraction in SSF [50, 51].

Also, the increase of the volume of extraction solvent to 7.5 mL g^{-1} of the dry solid substrate led to the improvement of lipase extraction, leading to a 1.5-fold increase in lipase activity extracted per gram of substrate, in comparison to the initial extraction conditions, without loss of enzyme final concentration in the extract obtained. Pal and Khanum [52] found an optimum solvent volume of 10 mL g^{-1} for the extraction of *A. niger* xylanase, while Díaz et al. [53] found that the use of 5 mL g^{-1} of solvent was most appropriate for extracting xylanase.

Consecutive extractions of the fermented substrate were also performed to determine the lipase recovery in the extraction, at optimum extraction conditions. Table 6 presents the lipase activity and the respective percentage of

Table 5 Conditions of extraction and respective lipase activity (LA)

Effect of extraction solvent type—5 mL g^{-1}, 2 h, 170 rpm, 20 °C		
Solvent (5 mL g^{-1})	LA ± SD (U g^{-1})	LA ± SD (U mL^{-1})
Distilled water	17 ± 1^{b}	3.0 ± 0.2^{ab}
Phosphate buffer	23 ± 1^{bc}	4.1 ± 0.2^{b}
10 g L^{-1} NaCl	5 ± 2^{a}	0.8 ± 0.3^{a}
10 g L^{-1} Tween 80	30 ± 3^{c}	5.2 ± 0.4^{b}
5 g L^{-1} Triton + 10 g L^{-1} NaCl	144 ± 5^{d}	25 ± 1^{c}
10 g L^{-1} Triton + 10 g L^{-1} NaCl	153 ± 6^{d}	27 ± 1^{cd}
5 g L^{-1} Triton	147 ± 9^{d}	26 ± 2^{c}
10 g L^{-1} Triton	175 ± 5^{e}	31 ± 1^{e}
20 g L^{-1} Triton	161 ± 4^{e}	29 ± 1^{de}
Effect of extraction volume—10 g L^{-1} Triton, 2 h, 170 rpm, 20 °C		
Volume (mL g^{-1})	LA ± SD (U g^{-1})	LA ± SD (U mL^{-1})
2.5	77 ± 3^{a}	28 ± 1^{a}
5	156 ± 3^{b}	27 ± 1^{a}
7.5	230 ± 6^{c}	28 ± 1^{a}

Values are the mean of triplicate analysis ± standard deviation (SD). Means with the same letter do not differ significantly at $p > 0.05$ (Tukey test)

Table 6 Results of lipase activity (LA) and respective lipase recovery (LR) from consecutive extractions of the fermented substrate

	Number of consecutive extractions				
	1st	2nd	3rd	4th	5th
LA ± SD (U g^{-1})	223 ± 5^{c}	65 ± 2^{b}	3.5 ± 0.2^{a}	0.9 ± 0^{a}	0.5 ± 0^{a}
LR ± SD (%)	76.1 ± 0.2^{d}	22.2 ± 0.3^{c}	1.2 ± 0.1^{b}	0.3 ± 0^{a}	0.2 ± 0^{a}

Values are the mean of triplicate analysis ± standard deviation (SD). Means within the same line with the same letter do not differ significantly at $p > 0.05$ (Tukey test)

lipase recovery of those experiments. The first extraction yielded a lipase activity of 223 ± 5 U g^{-1}, which corresponds to a lipase recovery of 76%. With a second extraction it was possible to extract almost all the remaining lipase contained in the fermented substrate. The results agreed with Rodriguez et al. [51] that obtained a lipase recovery of 70% in the first extraction, using 10 g L^{-1} Triton X-100.

Conclusions

The present study allowed to improve the lipase production by *A. ibericus* from OP mixed with WB, optimizing substrates' proportion, nitrogen source and lipase extraction. Optimum SSF conditions were achieved with the mixture of OP:WB (1:1), leading to a C/N mass ratio of 25.2 and 10.2% (w/w) lipids in substrate and supplemented with 1.33% (w/w) $(NH_4)_2SO_4$. The optimized medium was successfully tested in a laboratory-scale packed-bed bioreactor, a promising system for lipase production at industrial scale. Additionally, the important role of Triton X-100 on lipase extraction was proven.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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