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Aspergillus ibericus lipase production by solid-state fermentation of olive pomace

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ABSTRACT: Lipases are versatile catalysts with many applications, such as in food and detergents industries. Lipases can be produced by solid-state fermentation (SSF) using agro-industrial wastes. In addition, *A. ibericus* has been pointed as an interesting fungus to produce lipase through SSF of olive pomace (OP). The aim of this work was to optimize the production of lipase by *A. ibericus* under SSF using OP and wheat bran (WB). Additionally, extraction conditions of lipase were optimized. At optimum conditions lipase production and recovery improved 2-fold, yielding 223 ± 5 U/g. Optimum SSF conditions were using 30 g in a ratio of 1:1 (dry basis) of OP and WB supplemented with 0.4 g $(NH_4)_2SO_4$ at 60% of moisture content and incubated at 30°C during 7 days. The extraction of lipase was improved using 7.5 mL/g solid residues of 1% Triton X-100 homogenized for 0.5 hour at 250 rpm and 24°C.

1 INTRODUCTION

Solid-state fermentation (SSF) has been used for the production of value-added products using agro-industrial wastes (Salihu et al. 2012). SSF is a fermentation technique which involves the culture of microorganism on moist solid supports (Pandey 2003). It is the preferred choice for growing filamentous fungi since it simulates their natural habitat.

Olive pomace (OP) is a sludgy waste generated by the olive oil two-phase extraction system. It is an acidic and very humid material, rich in organic matter, potassium, nitrogen and fats (Alburquerque et al. 2006), which can be valorized for the production of lipases while being treated.

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are a class of hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over oil water interface (Treichel et al. 2009). The interest in the production of lipases is associated with their applications as additives in food, fine chemicals, detergents, waste water treatment, cosmetics, pharmaceuticals, leather processing and biomedical assays (Salihu et al. 2012). In addition, lipases also have important application in the field of bioenergy, especially in biodiesel production. Microorganisms including bacteria, fungi and yeast that produce extracellular lipases are recognized as the preferred sources of these enzymes because their recovery from the culture broth is facilitated (Salihu et al. 2012).

Aspergillus species within the section *Nigri* are important in many biotechnological processes. Species such as *A. niger* have a GRAS status from the FDA and have been widely used by the bioindustry. *Aspergillus ibericus* is a new black *Aspergillus* species that was isolated from wine grapes (Serra et al. 2006), and is able to produce lipase on OP and wheat bran (WB) through SSF (Oliveira et al. 2013).

Thus, the aim of this work was to optimize the production of lipase by *Aspergillus ibericus* MUM 03.49 under SSF. The variables investigated were the source and concentration of phosphorus, nitrogen and carbon (mixtures of OP and WB). Finally, at optimum conditions, a profile of lipase production over time and productivity was performed. Additionally, lipase extraction conditions were optimized.

2 MATERIALS AND METHODS

2.1 *Microorganism and residues*

Aspergillus ibericus MUM 03.49 (MUM culture collection, Braga, Portugal) was used. The fungus was grown on malt extract agar (MEA) plates (2% (w/v) malt extract, 2% (w/v) glucose, 0.1% (w/v) peptone and 2% (w/v) agar) at 30°C for 7 days and stored at 4°C. Spore suspensions of the inoculum were prepared by adding peptone solution (0.1% (w/v) peptone and 0.001% (w/v) Tween 80) to plates cultures, and after agitation were transferred to a falcon. The spore concentration of the suspension was adjusted to 10^7 spores/mL. OP samples were collected from a two-phase olive mill plant in Vila Real, Portugal, during the 2012/2013 campaign, and stored at −20°C. OP presented 69% ± 1% of moisture content (wet basis). WB was purchased in a local supermarket.

2.2 *Optimization of residues composition*

SSFs were performed in cotton-plugged 500 mL Erlenmeyer flasks containing 30 g dry solid residues in a ratio of 1:1 (w/w, dry basis) of wet OP and WB. $NaNO_3$ (0.6 g/30 g) was added to supplement the residues (Oliveira et al. 2013). The mixture of OP with WB resulted in optimum moisture content between 57% and 60%, without the need for its adjustment.

Flasks were prepared, autoclaved at 121°C for 20 min, cooled, inoculated with 1 mL of inoculum suspension and incubated at 30°C during 7 days. After the incubation period the fermented residues were extracted has described below to obtain the enzymatic extracts.

Different sets of experiments were conducted to evaluate the effect of phosphorus, nitrogen and residues composition in the production of lipase by *A. ibericus* under SSF.

The phosphorus source studied was KH_2PO_4 and concentrations tested were 0, 0.15, 0.3 and 0.6 g/30 g. The nitrogen sources tested were $NaNO_3$, urea, NH_4Cl and $(NH_4)_2SO_4$ and concentration tested were 0, 0.3 g and 0.6 g/30 g. $(NH_4)_2SO_4$ was further evaluated at concentrations that ranged from 0 to 1 g/30 g because it was the nitrogen source with the most pronounced effect.

To evaluate de effect of carbon sources, different ratios of dried and ground OP:WB were tested (1:0, 4:1, 3:2, 1:1, 2:3, 1:4 and 0:1, w/w). Experiments containing only WB supplemented with different percentages of olive oil (0, 1, 2.5, 5 and 10%) were also performed. Moisture content was adjusted to 60%.

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

2.3 *Extraction and lipase determination*

The fermented residues were homogenized with 5 mL of 1% Triton X-100 per g of dried residues at 170 rpm and 24°C for 2 h, using a shaker. Homogenates were then centrifuged (3000 rpm and 10 min at 4°C) and filtered. Lipase activity was determined by a spectrophotometric method, using *p*-nitrophenyl butyrate in potassium phosphate 50 mM at pH 7.0 and 37°C for 15 min. The absorbance was measured at 405 nm. 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 residue (U/g).

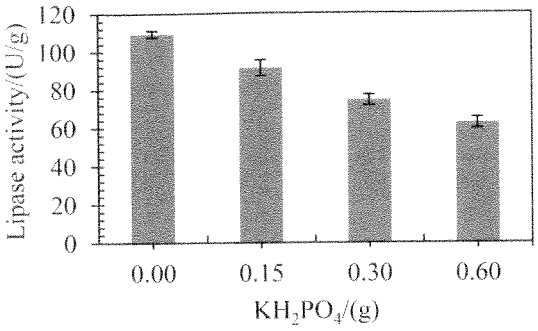

Figure 1. Effect of phosphorus source on lipase activity. Depicted values are the mean of triplicate analysis ± standard deviation.

2.4 *Optimization of lipase extraction conditions*

SSF were performed using the optimum conditions achieved. Initial conditions of extraction were 150 mL of extracting solvent, 2 h of shaking at 170 rpm and 24°C. Starting from this point, different variables were studied in order to improve lipase extraction. They were: type of solvent, volume of solvent, time of extraction, stirring rate and extraction temperature. Each of those variables was studied separately and following to the previous order. The best condition determined for each variable was used to optimize the following one by changing initial conditions. At the end of the lipase extraction optimization, consecutive extractions were performed in a same fermented residue in order to determine the extraction efficiency. The procedure was repeated 5 times, obtaining 5 extracts from the same fermented residue.

2.5 *Analysis of experimental data*

The data obtained were statistically analysed using SPSS (IBM SPSS Statistics, Version 22.0. Armonk, NY: IBM Corp.) in order to study the effect of variables on lipase production. 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%.

3 RESULTS AND DISCUSSION

3.1 *Optimization of residues composition*

In many cases, an additional source of phosphorus is important to induce an overproduction of some metabolite (Papagianni 2004). For example, Pérez-Rodríguez et al. (2014) found a positive effect of KH_2PO_4 on xylanase production by *A. niger* through SSF of corncob. In this work, the phosphorus source used (KH_2PO_4) produced a significant negative effect ($p < 0.0001$) on lipase production (Fig. 1). So, the mixture OP:WB seemed to supply enough phosphorus being unnecessary its supplementation.

Contrariwise, the nitrogen sources had a significant positive effect ($p < 0.001$) on lipase production (Table 1). All of them increased significantly the production of lipase when added to the OP:WB mixture, but the highest lipase activity was obtained with $(NH_4)_2SO_4$ at a concentration of 0.6 g/30 g of residues. Therefore, $(NH_4)_2SO_4$ was chosen to proceed with the following studies.

Figure 2 presents lipase yields of experiments conducted with different concentrations of $(NH_4)_2SO_4$. A significant effect ($p < 0.0001$) on lipase production was observed with increasing amounts of $(NH_4)_2SO_4$ until the maximum lipase activity of 138 ± 3 U/g was reached with a concentration of 0.4 g/30 g. This concentration was used in further SSFs.

Table 1. Lipase activity affected by the amount of different nitrogen sources. Values are the mean of triplicate analysis ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$.

	Lipase activity ± standard deviation/(U/g)			
Amount/(g)	$NaNO_3$	Urea	NH_4Cl	$(NH_4)_2SO_4$
0	89 ± 5			
0.3	111 ± 4[a]	128 ± 4	137 ± 3[b]	134 ± 5
0.6	115 ± 6[a]	106 ± 7	144 ± 5[b]	151 ± 7

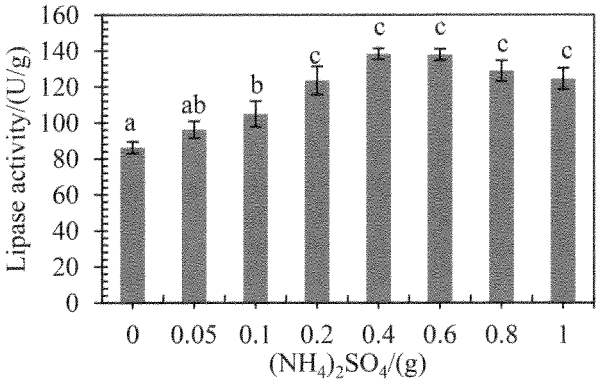

Figure 2. Profile of lipase activity as a function of the amount of $(NH_4)_2SO_4$. Depicted values are the mean of triplicate analysis ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$.

Lipase activity obtained for each tested OP:WB mixture is depicted in Figure 3a. The most favourable mixture for the production of lipases was the 1:1 ratio, yielding 144 ± 5 U/g of lipase activity. This yield corresponds to a 1.7-fold increase if compared to the SSF that contained only WB (0:1 ratio). It was also observed that *A. ibericus* could grow in all runs. However, it did not produce lipases in runs containing only OP or with low amount of WB (4:1 ratio). Mixed solid residues have been considered attractive for the growth of microorganisms on SSF, since they may act differently as support matrix, nutrient source and as inducers for the production of enzymes (Edwinoliver et al. 2010).

The effect of olive oil supplementation on WB was also studied to compare its effect with OP. It was observed that olive oil had a significant positive effect ($p < 0.0001$) on the production of lipases. An addition of 10% improved the production, reaching 152 ± 3 U/g (Fig. 3b). This activity was similar to one obtained with the OP:WB ratio of 1:1. Thus, the lipid content of OP can be a factor that is influencing most the production of lipases in experiments conducted with OP:WB mixtures. OP used in this work contained 10% of lipids, as determined by Oliveira et al. (2013). In this work, an OP:WB ratio of 1:1 was used in following studies.

Figure 4 presents results of lipase activity and its productivity over fermentation time. An increase of lipase production over time was observed, reaching 166 ± 5 U/g and 209 ± 10 U/g after 10 and 20 days of fermentation, respectively. However, the maximum productivity was obtained on the 6th day (1.8 ± 0.1 U/gh) with a lipase production of 127 ± 6 U/g.

3.2 *Optimization of lipase extraction conditions*

The effect of type of solvent, volume of solvent, time of extraction, stirring rate and extraction temperature on lipase recovery from fermented residues are presented in Table 2. It was found a

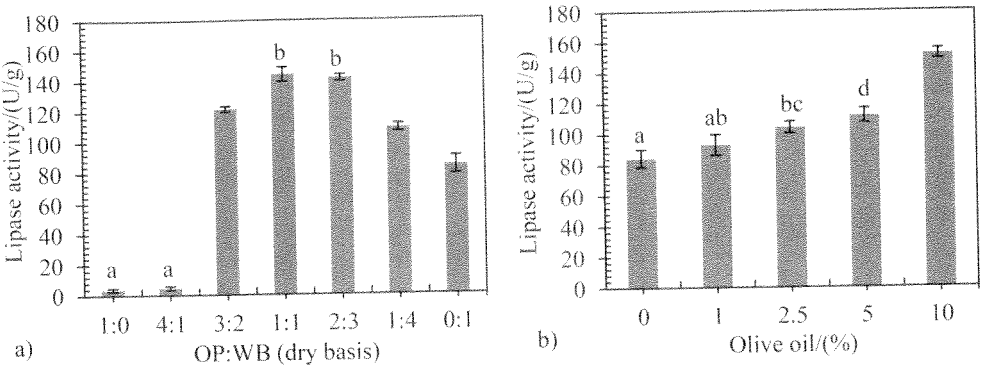

Figure 3. Results of lipase activity a) from SSF using different ratios of olive pomace (OP) with wheat bran (WB) and b) from SSF using WB with different concentrations of olive oil. Depicted values of lipase activity are the mean of triplicate analysis ± standard deviation. Means with the same letter do not differ significantly at $p > 0.05$.

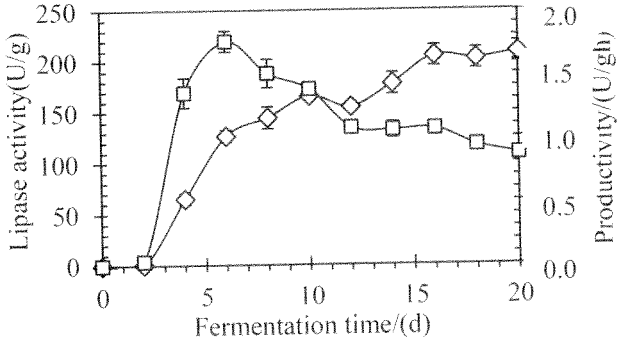

Figure 4. Profiles of lipase activity (—◇—) and its productivity (—□—) over fermentation time. Depicted values are the mean of triplicate analysis ± standard deviation.

Table 2. Conditions of extraction and respective lipase activity (LA). Values are the mean of triplicate analysis ± standard deviation (SD). Means with the same letter do not differ significantly at $p > 0.05$.

Solvent/ (5 mL/g)	LA ± SD/ (U/g)	Volume/ (mL/g)	LA ± SD/ (U/g)	Time/ (h)	LA ± SD/ (U/g)	Stirring/ rate/(rpm)	LA ± SD/ (U/g)	Temp./ (°C)	LA ± SD / (U/g)
Distilled water	17 ± 1^{a}	2.5	77 ± 3	0	127 ± 8	0	155 ± 5	24	218 ± 9^{a}
Phosphate buffer	23 ± 1^{ab}	5	156 ± 3	0.25	203 ± 6^{ab}	100	185 ± 8	26	217 ± 3^{a}
1% NaCl	5 ± 2	7.5	230 ± 6	0.75	217 ± 4^{a}	150	208 ± 7^{a}	30	133 ± 7^{b}
1%Tween 80	30 ± 3^{b}	10	198 ± 6^{a}	1	196 ± 4^{b}	200	205 ± 4^{a}	35	131 ± 4^{b}
1% Triton +		15	194 ± 7^{a}	2	189 ± 5^{b}	250	222 ± 7^{a}	40	176 ± 6
1% NaCl	153 ± 6^{c}	–	–	–	–	–	–	–	–
0.5% Triton	147 ± 9^{c}	–	–	–	–	–	–	–	–
1% Triton	175 ± 5^{d}	–	–	–	–	–	–	–	–
2% Triton	161 ± 4^{d}	–	–	–	–	–	–	–	–

Table 3. Results of lipase activity (LA) and respective extraction recovery (ER) from consecutive extractions of the fermented residue. Values are the mean of triplicate analysis ± standard deviation (SD).

	Number of consecutive extractions				
	1st	2nd	3rd	4th	5th
LA ± SD/(U/g)	223 ± 5	65 ± 2	3.5 ± 0.2	0.9 ± 0	0.5 ± 0
ER ± SD/(%)	76 ± 0	22 ± 0	1.2 ± 0.1	0.3 ± 0	0.2 ± 0

significant effect ($p < 0.0001$) of all variables on lipase extraction. The higher yields of lipase were recovered when the residue was extracted with 7.5 mL of 1% Triton X-100 per g of solid residue and the homogenization was done during 0.5 hour at 250 rpm and 24°C.

Consecutive extractions of fermented residues were also performed in order to determine the recovery of lipase at optimum extraction conditions. Table 3 presents lipase activity and the respective recovery percentages of those experiments. The first extraction yielded a lipase activity of 223 ± 5 U/g, which corresponds to an extraction recovery of 76 ± 0%. With a second extraction it was possible to extract almost all the lipase contained in the residue. The results agree with Rodriguez et al. (2006) who obtained a lipase recovery of 70% in the 1st extraction, using 1% Triton X-100.

This study shows that OP and WB are suitable residues to produce *A. ibericus* lipase under SSF. Mixtures of these agro-residues can be used without being necessary any processing step such as drying or grind. They can be applied directly without moisture adjustment, requiring only the addition of 0.4 g of $(NH_4)_2SO_4$ per 30 g of solid residues. With this study, an increase in lipase production of 2-fold was achieved.

4 CONCLUSIONS

In conclusion, it was observed that the addition of phosphorus did not improve the production of lipase by *A. ibericus*. On the contrary, the nitrogen source influenced positively lipase production. The best nitrogen source was found to be ammonium sulphate. Additionally, the carbon source and its composition influenced substantially its production. The mixture of OP with WB in a 1:1 ratio was found to be the most favourable composition. It was also observed that the recovery of lipase from residue is improved when the extraction is performed using 1% Triton X-100 (7.5 mL/g) at 250 rpm and 24°C during 30 min. Therefore, the production of lipase through SSF of OP with WB using *A. ibericus* is an interesting strategy for OP valorization.

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