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VALORISATION OF OLIVE POMACE BY SOLID-STATE FERMENTATION WITH *ASPERGILLUS* SPECIES FOR LIPASE PRODUCTION

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ABSTRACT
Pollution by olive mill wastes is a crucial problem in Mediterranean area and their proper management and utilization is demanded. Olive pomace offers excellent properties to produce enzymes by solid-state fermentation (SSF) using filamentous fungi. Particularly for lipase production, since it has residual content of olive oil.
The aim of this work was to optimize the production of lipase by *Aspergillus ibericus* MUM 03.49, *Aspergillus niger* MUM 03.58 and *Aspergillus tubingensis* MUM 06.152, under SSF of olive pomace. A Taguchi L-9 orthogonal array based on 4 factors at 3 levels (ratio between olive pomace and wheat bran (OP:WB), $NaNO_3$, Czapek nutrients and time) was implemented. SSF was carried out in Erlenmeyer flasks of 500 mL and lipase activity was measured using *p*-nitrophenyl butyrate as substrate.
Results showed for all fungi that the factor with most significant effect on lipase production was the mixture OP:WB, concluding that the presence of wheat bran on substrate favored lipase production. $NaNO_3$ concentration and time presented some effect and presence of Czapek nutrients did not added significant advantages on lipase production. *A. ibericus* was the best lipase producer, being a promising microorganism for lipase production. Under optimized conditions it produced 20.78 U/gds of lipase.

Keywords: olive pomace, solid-state fermentation, *A. ibericus*, *A. niger*, *A. tubingensis*, lipase

INTRODUCTION
Two-phase olive mill waste is a sludgy waste generated by the olive oil two-phase extraction system. It is also known as olive pomace (OP), alperujo, olive wet husk or olive wet cake. Olive pomace is composed by olive stone, pulp and skin, water (50-70%) and oil residues (4-18%) [1]. Olive pomace is presently the most important residue produced by olive oil industry because the two-phase extraction system comes to be largely implemented in new olive mills. Therefore, strategies for its treatment and valorization are currently necessary. The biotechnological valorisations most studied include the production of biofuels such as methane, ethanol or hydrogen, the production of biopolymers and enzymes, such as lipases [2]. Recently, a growing interest on lipase production in low-cost agro-industrial wastes has emerged, and the use of substrates such as wheat bran has been proposed [3]. Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are a large class of enzymes that act on carboxylic ester bonds. Lipases from microbial sources are the most used for biotechnological applications. They are obtained from bacteria, yeasts and filamentous fungi through submerged and solid-state fermentation processes, using wild or recombinant strains [4]. The black aspergilli are an important group of food spoilage fungi but they also have a relevant importance in biotechnology. Most recognized species is undoubtedly *Aspergillus niger*, which grants the GRAS status from FDA. *Aspergillus ibericus* is a new species from this group of fungi that has been isolated from wine grapes [5]. In previous studies, it has been demonstrated that it is a good producer of lipases. On a 2-L bioreactor, *A. ibericus* produced up to 8.319 U/L of lipase using olive mill waste water [6].

The aim of this work was to optimize the production of lipase by *Aspergillus ibericus* MUM 03.49, *Aspergillus niger* MUM 03.58 and *Aspergillus tubingensis* MUM 06.152, under SSF of olive pomace. The optimization of the process was performed evaluating parameters such as substrate composition, nitrogen source concentration, mineral nutrients and time of fermentation.

MATERIALS AND METHODS

Olive pomace characteristics

OP samples were collected from a local two-phase olive mill and stored at -20 ºC to be used throughout the study. OP characteristics were determined by performing analysis in triplicate as follows. Moisture content was determined by weight loss after drying overnight at 105 ºC and ash by ignition loss at 550 ºC for 5 h. Total organic content was calculated by making the difference between the dry and ash weight. Total nitrogen content was determined using the Hach Lange kit LCK338. Total organic carbon content was determined using the Hach Lange kit LCK386. The pH, soluble reducing sugars, phenolic and protein content were determined in water extracts (2:10, w:v). The concentration of the reducing sugars was determined with a DNS-adapted method [7]. The amounts of the phenols were determined with the Folin-Ciocalteu method with tyrosol as the standard [8]. The protein content was determined with the Bradford method with BSA as the standard [9]. Lipids were extracted from dried samples with diethyl ether in a Tecator Soxtec system (model: HT2 with 1045 extraction unit and 1046 service unit) and weighed. Cellulose, hemicelluloses and lignin content were determined quantifying glucose, xylose and arabinose by HPLC after acid hydrolysis in a two-stage acid treatment that involved: 1st - hydrolysis with 72 wt% H_2SO_4 at 30 ºC for 1 h; 2nd - dilution of the media to 4 wt% H_2SO_4 and hydrolysis at 121 ºC for 1 h [10].

Biological material

Aspergillus ibericus MUM 03.49, *Aspergillus niger* MUM 03.58 and *Aspergillus tubingensis* MUM 06.152 (MUM culture collection, Braga, Portugal) were used. They were revived on malt extract agar (MEA) plates (2% malt extract, 2% glucose, 0.1% peptone and 2% agar) from a frozen glycerol stock. Spore suspensions were prepared by vortex-mixing seven-day-old culture slants with 4 mL of peptone solution (0.1% peptone and 0.001% Tween 80). The spore concentration of the suspension was adjusted to 10^6 spores/mL using a Neubauer counting chamber and used as inoculum.

SSF preparation, extraction and lipase determination

Solid-state fermentations were performed in cotton-plugged 500 mL Erlenmeyer flasks containing 30 g of dried substrate. Moisture was adjusted to 75% (wet basis) with distilled water. Flasks were autoclaved at 121 °C for 15 min, cooled, inoculated with 1 mL of inoculum suspension and incubated at 25 ºC, according to conditions of each experiment.

At the end of the incubation period, fermented substrates were homogenized with 150 mL of 1% NaCl and 0.5% Triton X-100 at 170 rpm and 4 ºC for 2 h using a shaker. Homogenates were then centrifuged (12000 *g* and 10 min at 4 °C) and filtered using a Whatman Nº 1 filter paper. The resulting enzymatic extracts were preserved at 4 ºC until lipase determination.

The extracellular lipase activity was determined by colorimetric assay, using *p*-nitrophenyl butyrate (*p*-NPB) as described by Gomes et al. [11]. One unit of lipase activity (U) was expressed as the amount of enzyme that produced 1 µmol of *p*-nitrophenol per minute under the assay conditions. All the analyses were performed in triplicate.

SSF of olive pomace for lipase production under different conditions

A Taguchi L9 orthogonal array was designed using Qualitek-4 software (Nutek Inc., USA) in order to study the effect of factors on lipase activity. Three levels were defined for four factors: ratio of olive pomace:wheat bran (1:0; 2:1 and 1:1, w/w), supplementation with $NaNO_3$ (0.15; 0.3 and 0.6 g), supplementation with mineral nutrients (Czapek nutrients) (0 - no supplementation; 1X – 1 g/L K_2HPO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L $CaCl.2H_2O$, 0.01 g/L $FeSO_4.7H_2O$, 0.01 g/L

$ZnSO_4 \cdot 7H_2O$ and 0.005 g/L $CuSO_4 \cdot 5H_2O$; 2X – same as 1X but with concentration doubled); and fermentation time (7; 14 and 21 days).
Functions "Standard analysis" using "Average of results" and "bigger is better" were used to evaluate the contribution of the factors on lipase activity and to determine the optimum SSF conditions. Qualitek-4 was also used to perform the analysis of variance (ANOVA) of the results obtained.

RESULTS AND DISCUSSION

Olive pomace characteristics
OP used had the following characteristics (mean values ± SD; n = 3): pH, 4.9 ± 0.02; moisture content, 74.8 ± 0.6%; total organic content, 0.24 ± 0.08 g/gds; total nitrogen, 1.9 ± 0.6 mg/gds; total organic carbon, 0.5 ± 0.02 g/gds; reducing sugars, 24.3 ± 1.4 mg/gds; phenols, 2.6 ± 0.04 mg/gds; protein, 0.3 ± 0.03 mg/gds; lipids, 10.0 ± 0.4%; cellulose, 0.07 ± 0.01 g/gds; hemicelluloses, 0.04 ± 0.01 g/gds; lignin, 0.58 ± 0.01 g/gds and ash, 1.0 ± 0.07%. The values are in accordance with OP characteristics reported elsewhere [1, 12, 13].

SSF of olive pomace for lipase production under different conditions
Lipase activities obtained ranged from 0 to 18.73 U/gds, as presented in Table 1. *Aspergillus* species were able to grow in all runs performed, in spite of olive pomace contain organic acids, phenolic compounds and fats that may have negative effect on microbial activity [12]. However, in runs without wheat bran as substrate, lipase activity was, in general, null for all species. The fungus *A. ibericus* produced a maximum lipase activity of 18.73 U/gds at conditions of 1:1 OP:WB, 0.6 g $NaNO_3$, 1X mineral nutrients and 7 days. *A. niger* presented a maximum lipase activity of 10.92 U/gds at conditions of 1:1 OP:WB, 0.3 g $NaNO_3$, 0 mineral nutrients and 21 days; and *A. tubingensis* a value of 5.06 U/gds at conditions of 2:1 OP:WB, 0.15 g $NaNO_3$, 1X mineral nutrients and 21 days.
Mixed solid substrates are 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 [14]. For example, Kumar et al. reported an optimum ratio of 1:1 of grease and wheat bran for lipase production (38 U/mL) by *Penicillium chrysogenum* concluding that the fungi first utilizes wheat bran for mycelia growth and then the grease waste [15]. This combination of different substrates may favor fungal development and the production of foreseen enzymes. Effectively, we also observed that the addition of wheat bran to OP could improve the production of lipase by all tested species.
The obtained maximal lipase activities were found to be higher than those obtained by other researchers, which also used SFF and filamentous fungi to produce lipase. Falony et al. obtained a lipase activity of 9.14 U/g using SSF of *A. niger* in wheat bran at 65% moisture content, 1.5% olive oil and other optimized conditions [16].

Table 1 – Factors and assigned levels in Taguchi L9 orthogonal array and experimental values of lipase activity obtained for the different fungi.

					Experimental values of lipase activity ± SD/(U/gds)		
Run	OP:WB	$NaNO_3$/(g)	Czapek nutrients	Time/(d)	*A. ibericus*	*A. niger*	*A. tubingensis*
1	1:0	0.15	0	7	0.00 ± 0.00	0.24 ± 0.01	0.13 ± 0.03
2	1:0	0.3	1X	14	0.02 ± 0.03	0.50 ± 0.04	0.11 ± 0.01
3	1:0	0.6	2X	21	1.76 ± 0.53	0.19 ± 0.01	0.22 ± 0.15
4	2:1	0.15	1X	21	0.98 ± 0.34	10.56 ± 0.36	5.06 ± 0.59
5	2:1	0.3	2X	7	1.40 ± 0.30	2.27 ± 0.56	1.40 ± 0.27
6	2:1	0.6	0	14	4.20 ± 0.58	10.92 ± 1.86	1.87 ± 0.42
7	1:1	0.15	2X	14	11.92 ± 1.01	9.82 ± 1.45	1.81 ± 0.60
8	1:1	0.3	0	21	13.16 ± 1.05	10.92 ± 1.53	3.65 ± 0.31
9	1:1	0.6	1X	7	18.73 ± 0.31	4.25 ± 1.24	1.33 ± 0.42

Guatarra et al. obtained a lipase production of 19.6 U/g in 72 h at 30 ºC by growing *Penicillium simplicissimum* in babassu cake supplemented with sugar cane molasses and moistened to 70% [17]. On the contrary, obtained lipase activities were lower than other reported by the following authors. Kamini et al., which worked with *A. niger* on different substrate, found gingelly oil cake to be the best substrate obtaining a lipase activity of 363.6 U/g under optimum conditions [18]. Edwinoliver et al. reported maximum lipase activity of 459.1 U/g growing *A. niger* in a combination of 1:3 of coconut oil cake and wheat bran at 30 ºC during 96 h [14].

The effect of factor OP:WB ratio on lipase activity is presented in Figure 1. As it can be seen, the presence of wheat bran on substrate could improve lipase production. For the fungus *A. ibericus*, lipase activity was clearly affected by OP:WB ratio (1:1, w/w) and by $NaNO_3$ (addition of 0.6 g in flasks). Contrariwise, the mineral nutrients and time did not present a clear influence on lipase activity produced by this fungus. Lipase of *A. niger* was also affected greatly by OP:WB ratio with 1:1 being the best ratio to improve the activity. Also, ratio of 2:1 presented good results (Figure 1b). Further, the enzyme production was affected by time with 21 or 14 days being the best for lipase production. $NaNO_3$ and Czapek nutrients presented lower effect with 0.15 g of $NaNO_3$ and absence of Czapek nutrients improving lipase production. Lipase of *A. tubingensis* was, similarly to the other fungi, affected by OP:WB ratio, with 2:1 being the best ratio. In this case, time also affected the production of the enzyme, with 21 days being the best fermentation time. $NaNO_3$ and Czapek nutrients presented lower effects but lipase production was improved when 0.15 g of $NaNO_3$ and 1X of Czapek nutrients were used.

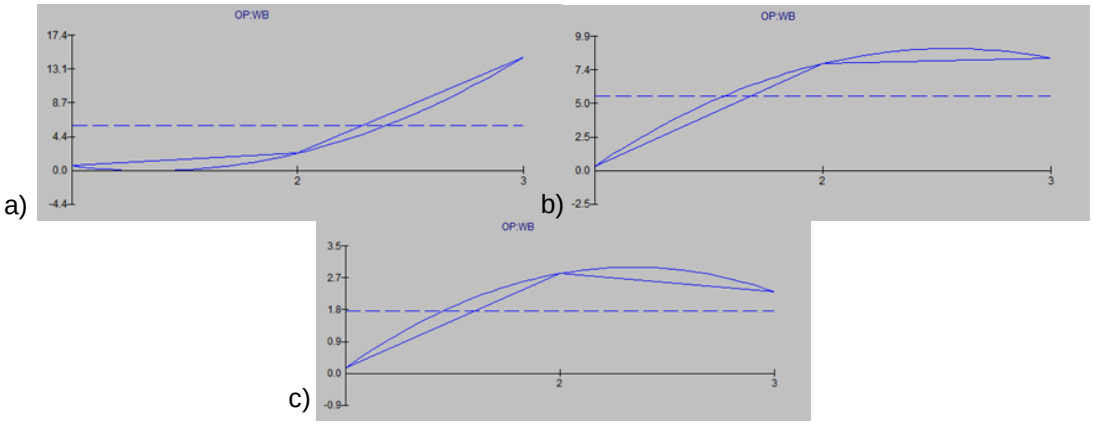

Figure 1 – Effect of the factor OP:WB ratio on lipase activity of a) *A. ibericus* MUM 03.49; b) *A. niger* MUM 03.58 and c) *A. tubingensis* MUM 06.152, using Taguchi L9 orthogonal array.

Table 2 – Analysis of variance (ANOVA) for the Taguchi L9 orthogonal array.

Fungus	Factor	Sum of squares	Variance	F-Ratio	Percent P/(%)
A. ibericus	OP:WB	1058.46	529.23	1568.36	90.55
	$NaNO_3$	81.47	40.73	120.71	6.92
	Czapek nutrients	10.83	5.42	16.05	0.87
	Time	11.33	5.66	16.79	0.91
	Error	6.07	0.34	-	0.75
A. niger	OP:WB	244.68	122.34	111.28	60.36
	$NaNO_3$	17.47	8.74	7.95	3.80
	Czapek nutrients	33.64	16.82	15.30	7.83
	Time	96.06	48.03	43.69	23.36
	Error	9.89	1.10	-	4.66
A. tubingensis	OP:WB	34.79	17.40	124.74	49.25
	$NaNO_3$	6.39	3.19	22.90	8.72
	Czapek nutrients	5.01	2.51	17.98	6.76
	Time	21.37	10.69	76.63	30.1
	Error	2.51	0.14	-	5.18

Similarly to this work, where Czapek nutrients did not present significant influence on lipase production, Kumar et al. reported that Czapek-dox medium, used as salts and moisture facilitator for fungal growth, contributed to decrease lipase activity [15]. It is also known that high concentration of nitrogen source in media is effective in enhancing the production of lipases by microorganisms [19]. For example, Sun and Xu reported that some nitrogen sources such ammonium hydrogen phosphate had positive effect on lipase production by *Rhizopus chinensis*, and that others did not [19]. In our experiments, $NaNO_3$ had positive effect on lipase production of *A. ibericus*. This work also showed that the optimum fermentation time for lipase production is dependent on the fungus used.
Analysis of variance presented in Table 2 shows that OP:WB ratio was the factor most significant for lipase production, since it presented the highest F-ratio values, and a percent of influence of 49.25% for *A. tubingensis*, 60.36% for *A. niger* and 90.55% for *A. ibericus*. According to F-ratio and percent of influence, the second factor with most pronouncing effect on lipase activity was $NaNO_3$ for *A. ibericus* and time for *A. niger* and *A. tubingensis*. Error was low, indicating good reproducibility of experiments.

Optimization of conditions to produce lipase
Table 3 presents optimum fermentation conditions in order to improve lipase activity for the three *Aspergillus* species. Also, it presents the expected lipase activity in those conditions calculated using the "bigger is better" function of Qualitek-4.

Table 3 – Optimum level of factors and expected lipase activity at optimum conditions, using Taguchi L9 orthogonal array.

Fungus	OP:WB	$NaNO_3$/(g)	Czapek nutrients	Time/(d)	Current average/ (U/gds)	Expected result/ (U/gds)
A. ibericus	1:1	0.6	1X	7	5.80	18.73
A. niger	1:1	0.15	0	21	5.52	13.23
A. tubingensis	2:1	0.15	1X	21	1.73	5.05

It was expected a production of 18.73 U/gds for *A. ibericus*, 13.23 U/gds for *A. niger* and 5.05 U/gds for *A. tubingensis*. The current average of values of lipase activity was similar for *A. ibericus* and *A. niger*, around 6 U/gds, but much smaller for *A. tubingensis*, 1.73 U/gds.changes in the level of conditions were done taking into account their effect on lipase activity. For *A. ibericus* and for *A. tubingensis*, we chose not to add the Czapek nutrients because they did not influence the lipase production. For *A. niger*, OP:WB ratio of 2:1 and 14 days of fermentation were selected rather than OP:WB ratio of 1:1 and time of 21 days as presented in Table 3, because it was more convenient to use more OP and to reduce the fermentation time.
Results of lipase activity in optimum fermentations conditions were similar to the expected results (Table 4 and Table 3, respectively).

Table 4 – Results of lipase activity in best conditions achieved.

Fungus	OP:WB	$NaNO_3$/(g)	Czapek nutrients	Time/(d)	Lipase activity/ (U/gds)
A. ibericus	1:1	0.6	0	7	20.78
A. niger	2:1	0.15	0	14	10.10
A. tubingensis	2:1	0.15	0	21	5.87

A. ibericus was the best lipase producer, achieving 20.78 U/gds. *A. niger* and *A. tubingensis* presented lipase activity of 10.10 U/gds and 5.87 U/gds, respectively.

CONCLUSIONS
A. ibericus is a promising microorganism for lipase production and OP mixed with other materials as wheat bran can be used as substrate on SSF for lipase production. Further studies should be performed in order to improve lipase production.

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