Wastes: Solutions, Treatments and Opportunities – Vilarinho, Castro & Russo (eds)
© 2015 Taylor & Francis Group, London, ISBN 978-1-138-02882-1

Scale-up of *aspergillus ibericus* lipase production by solid-state fermentation

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ABSTRACT: This work deals with the production of lipase by *Aspergillus ibericus* under Solid State Fermentation (SSF) of Olive Pomace (OP) with Wheat Bran (WB) at packed-bed, tray-type and pressurized bioreactors. Moreover, the effect of the sterilization step of the residues on lipase activity was studied. The sterilization at 121°C of residues benefits lipase production. SSF scale-up from flasks to different bioreactors was successfully performed with a slight lipase production decrease from 27% to 40% of the total lipase units produced per mass of residue in Erlenmeyer's flasks using autoclaved residues (183 ± 21 U/g). Contrariwise, results of specific activity obtained in flasks (51 ± 4 U/mg) were similar to the obtained in the packed-bed bioreactor (using autoclaved residues) and in the pressurized bioreactor (using no autoclaved residues).

1 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 (Pandey 2003, Singhania et al. 2009). Due to low water activity in SSF, lower demand on sterility is needed (Singhania et al. 2009). Also, SSF offers the opportunity of the utilization of low cost agro-industrial residues for metabolites production while treating them (Pandey 2003).

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 and nitrogen, which also contains water-soluble carbohydrates, phenols and fats (Alburquerque et al. 2006), offering excellent properties to produce enzymes, particularly lipase, since it has residual content of olive oil. Previous work has demonstrated the ability of *Aspergillus ibericus* MUM 03.49 for the production of lipase (Oliveira et al. 2013) under SSF of OP with wheat bran (WB), and also under submerged fermentation of olive mill wastewaters (Abrunhosa et al. 2013).

In SSF processes, microorganism's growth may be limited by heat transfer and/or mass transfer of oxygen or nutrients, depending on the location in the substrate bed, the stage of the fermentation, and the design and operation of the bioreactor (Mitchell et al. 2000). Different bioreactor types have been used in SSF, including tray-type, packed-bed and horizontal rotary-drum, presenting their own advantages and disadvantages (Singhania et al. 2009).

The aim of this work was to scale-up the production of lipase from *Aspergillus ibericus* MUM 03.49 by SSF of OP with WB, studied in previous works on Erlenmeyer's flasks. (Oliveira et al. 2013). Packed-bed, tray-type and pressurized bioreactors were used and SSF conditions were optimized. Also, studies of the effect of the sterilization of residues on lipase production were performed.

2 MATERIALS AND METHODS

2.1 *Microorganism and residues*

Aspergillus ibericus MUM 03.49 (MUM culture collection, Braga, Portugal) was used. *A. ibericus* is a black *Aspergillus*, isolated from wine grapes in Portugal and in Spain (Serra et al. 2006). 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 *SSF of OP with WB*

SSFs experiments were performed using OP mixed with WB. SSF conditions of lipase production were as follows: ratio of 1:1 (w/w, dry basis) of OP with WB, 0.0133 g/g of $(NH_4)_2SO_4$, 33.33 μL/g of 10^7 spores/mL inoculum suspension. The mixture of OP with WB resulted in optimum moisture content between 57% and 60%, without the need for its adjustment. Fermentations were carried out at 30°C during 7 days without agitation. SSF was performed with autoclaved (121°C, 200 kPa for 20 min) and also with non-autoclaved residues.

2.2.1 *Lipase production in 500 mL Erlenmeyer's flasks*

SSFs were carried out in cotton-plugged 500 mL Erlenmeyer's using 30 g of residues (OP + WB). SSF were performed in triplicate, where three flasks containing residues were autoclaved and another three were not. They were cooled, inoculated and incubated at conditions previous described. Also, controls were performed using the residues without autoclaving and without inoculating the fungus.

2.2.2 *Lipase production in packed-bed bioreactor*

The packed-bed bioreactor consisted in 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 0.45 μm cellulose filter and bubbled in distilled water before to enter in the column. The air flow was measured and controlled by a flowmeter (Aalborg Instruments & Controls, Inc., USA). The air outlet was bubbled in 1 M NaOH. The bioreactor and residues were previously autoclaved. The column was completely filled with 25 g of residue inoculated. SSF were performed at different aeration rates of 0.05 L/min, 0.1 L/min and 0.2 L/min. Experiments were performed in triplicate. Also, an additional SSF without aeration was performed.

2.2.3 *Lipase production tray-type bioreactor without forced aeration*

The tray-type bioreactor used consisted in a vertical incubator (112 × 48 × 45 cm) containing four stainless steel trays (38 × 26 × 5 cm). SSF was carried out in the 4 trays of the bioreactor at the same conditions. The residue was previously autoclaved. Each tray was filled with 300 g of residue, cooled and inoculated, resulting in a bed height of 2.5 cm. The incubator was opened once a day for monitoring and to allow aeration. SSF monitoring included temperature measurement and weight loss in each tray.

2.2.4 *Lipase production in pressurized bioreactor*

SSF was carried out in 19 dm^3 (42 cm height and 24 cm diameter) stainless steel stirred tank bioreactor, a pressurized bioreactor (4555, Parr Instrument Company, USA). SSF was performed using 500 g of residue no autoclaved. The bed height formed was around 8 cm. Air pressure of 200 kPa, 400 kPa and 700 kPa, was selected by the inlet air pressure setting and by controlling the regulatory outlet air valves. Also, different aeration rates were tested, 1 L/min and 2 L/min of outlet gas, at different pressures. SSFs were performed in duplicate.

2.3 *Lipase and protein extraction and determination*

Before extraction, a representative amount of the fermented residue was taken for moisture content determination. The fermented residues were homogenized with 5 mL of 1% Triton X-100 per g of dried residue at 170 rpm and 25°C for 2 h, using a shaker. Homogenates were then centrifuged (3000 rpm and 10 min at 4°C) and filtered. The enzymatic extracts were preserved at 4°C.

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).

The protein content was determined by Bradford's method, using BSA as the standard (Bradford 1976). Protein concentration was expressed as mg of protein per gram of dry solid residue (mg/g). Specific activity was obtained by the ratio between lipase activity and protein concentration. It was expressed as units of lipase activity per mg of total protein (U/mg). All analyses were performed in triplicate.

2.4 *Analysis of experimental data*

The data obtained were statistically analysed using SPSS (IBM SPSS Statistics, Version 22.0. Armonk, NY: IBM Corp.). 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 *Erlenmeyer's flasks*

SSF were performed in Erlenmeyer's flaks with and without autoclaving the residues. The growth of A. ibericus was observed in both residues, where the prevalence of A. ibericus in the no autoclaved residues was observed, presenting black spores, while in the control experiment different green and brown spores were observed.

In what concern moisture content, there were no significant differences between the results of SFFs. With respect to the pH of the fermented residues, lower pH was obtained autoclaving the residues. As expected, autoclaving the residues eliminates the wild microbial population, contributing to a better colonization with the *Aspergillus* strain used as inoculum. Since black *Aspergillus* species are known producers of many organic acids, a significant decrease of the final pH was observed (from 4.8 ± 0.1 to 4.3 ± 0).

Similarly, a significantly higher lipase production was obtained when autoclaving the residue. Autoclaving the residue presented significant positive effect ($p < 0.01$) on lipase produced per mass of residue and on specific activity (units per mass of total protein), reaching 183 ± 21 U/g and 51 ± 4 U/mg, respectively. For another hand, residues sterilization led to the decrease of protein concentration (3.6 ± 0.3 mg/g) and, consequently, the specific activity was significantly higher. In the flasks using no autoclaving residues a lipase production and specific activity of 121 ± 11 U/g and 31 ± 2 U/mg, respectively, was obtained.

3.2 *Packed-bed bioreactor*

Different aeration rates were evaluated in a packed-bed bioreactor and results are presented in Figure 1. It was observed the dehydration of the residue at the beginning of the column over fermentation time, even using saturated air. Aeration rate presented significant effect ($p < 0.0001$) on lipase activity. Maximum lipase production and specific activity was obtained at 0.05 L/min, reaching 134 ± 2 U/g and 50 ± 13 U/mg, respectively.

The aeration rate favours the transport of oxygen to the solid residue, however, above the optimal aeration, lipase activity may decrease due to the fungal metabolism changes (Díaz et al. 2013).

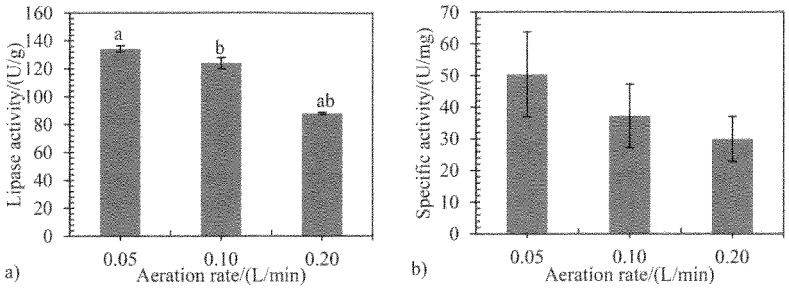

Figure 1. Effect of aeration rate on a) lipase production and b) specific activity of SSF in packed-bed bioreactor. Depicted values are the mean of three independent fermentation experiments ± standard deviation. Means with the same letter differ significantly at $p < 0.05$.

Pérez-Rodríguez et al. (2014) found an optimum aeration rate of 0.1 L/min in a packed-bed bioreactor using 20 g corncob for optimization of xylanase production under SSF with *A. niger*. A SSF without aeration was performed, where no fungal growth was observed.

Results of lipase obtained in packed-bed suffered a slight decrease of 27% of the lipase produced in flasks. However, the specific activity was similar (50 ± 13 U/mg) to the obtained in flasks. The slight reduction of lipase could be explained by the dehydration along the column derived from the aeration used.

3.3 *Tray-type bioreactor*

SSF in the tray-type bioreactor was performed using autoclaved residues without weight loss control. The weight loss of the trays was decreasing with time reaching 53% at the end of fermentation time. As a consequence, the moisture content of the fermented residues decreased to minimum values of 27% ± 3%, as shows Table 1. Lipase suffered a drastic decrease comparing to the values obtained in flasks, yielding 74 ± 10 U/g and 22 ± 2 U/mg, of lipase per g of residues and of specific activity, respectively.

Since the final moisture content was too low, another SSF was performed at similar conditions, but with control of weight loss by adding distilled water once a day, in the same amount of lost weight. Also, a SSF with no autoclaved residue with control of weight loss was performed. In all the fermentations, it was observed the increase of residues temperature till 34.3°C on the first and/or second day, due to the fungus's growth, after that the temperature was decreasing with fermentation time till 30°C. In both SSF with weight loss control, the weight loss at the end of fermentation was only 9%.

It was observed significant differences ($p < 0.005$) on lipase obtained. Lipase production was higher in SSF with weigh loss control, reaching 111 ± 27 U/g and 131 ± 16 U/g, using autoclaved and no autoclaved residues, respectively (Table 1). With respect to the specific activity, conditions used presented significant effect ($p < 0.005$) on specific activity. Similarly to the SSF in flasks, the specific activity was higher on SSF with autoclaved residue with weight loss control (36 ± 6 U/mg) than in SSF without autoclaving the residue. These results demonstrated the importance of moisture control to improve lipase production and its specific activity, and again the sterilization of the residues to improve the specific activity.

At a tray-type bioreactor, with moisture control, a decrease from 28% to 40% of the total production in flasks (183 ± 21 U/g) was observed. And also, a reduction in specific activity between 29% and 52% of that in flasks was obtained. This reduction could be attributed to the dehydration of the residue over fermentation time, high bed height (2.5 cm) used and the residues temperature rise during the culture growth. For example, Vaseghi et al. (2013) determined an optimum bed height of 0.5 cm for SSF on tray-type bioreactor.

Table 1. Results of moisture content (MC), pH, lipase activity (LA), specific activity (SA) and protein concentration (PC) in tray-type bioreactor at different conditions. Values are the mean of four trays of the incubator ± standard deviation (SD). Means with the same letter differ significantly at $p < 0.05$.

Condition	MC ± SD/(%)	pH ± SD	LA ± SD/(U/g)	SA ± SD/(U/mg)	PC ± SD/(mg/g)
Autoclaved without weight loss control	27 ± 3^{ab}	4.5 ± 0^{a}	74 ± 10^{a}	22 ± 2^{a}	3.3 ± 0.2^{a}
Autoclaved with weight loss control	66 ± 3^{a}	4.4 ± 0^{b}	111 ± 27	36 ± 6^{ab}	3.0 ± 0.3^{b}
No autoclaved with weight loss control	67 ± 3^{b}	5.0 ± 0.1^{ab}	131 ± 16^{a}	25 ± 5^{b}	5.4 ± 0.3^{ab}

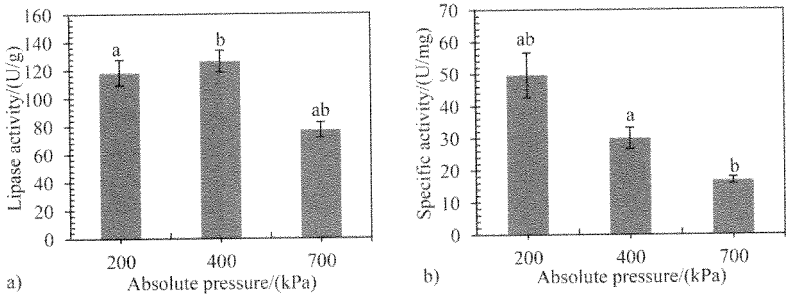

Figure 2. Results of a) lipase activity and b) specific activity of SSF in a pressurized bioreactor at different absolute air pressure and at aeration rate of 1 L/min. Depicted values are the mean of two independent fermentation experiments ± standard deviation. Means with the same letter differ significantly at $p < 0.05$.

3.4 *Pressurized bioreactor*

Pressurized air can be successfully applied to cultivations, as a way of improving the oxygen transfer rate to aerobic cultures (Belo et al. 2003). However, above certain limits the increased air pressure and the consequent increase in oxygen partial pressure may have detrimental effects on cells activity and product formation (Belo et al. 2005). Lopes et al. (2009, 2008) found significant improvements on lipase productivity from a nonconventional yeast under submerged fermentation, using air pressure up to 600 kPa.

Figure 2 presents results of SSF in a pressurized bioreactor, at aeration rate of 1 L/min and at different air pressures. Air pressure significantly affected ($p < 0.01$) lipase production. Maximum lipase activity was found at 400 kPa (126 ± 8 U/g). However, the specific activity was higher at 200 kPa (50 ± 7 U/mg). SSF at 2 L/min of aeration rate and at different air pressure were performed (data not shown). Similar to the results of SSF at 1 L/min, maximum lipase production and specific activity was obtained at 200 kPa (125 ± 5 U/g and 42 ± 4 U/mg, respectively). Results of SSF did not present significant differences using aeration rate of 1 L/min or 2 L/min.

In comparison to results from flasks, a decrease in 31% of lipase produced in pressurized bioreactor at 200 kPa and 1 L/min was observed. Contrariwise, the specific activity was similar to the flasks, even using no autoclaved residue (50 ± 7 U/g). The pressure of 200 kPa led to lower production of protein and consequently to maximum specific activities, presenting similar effects to the autoclaving residue.

4 CONCLUSIONS

A. ibericus presented good performance of growth and lipase production using no sterilized residue. For another hand, the effect of autoclaving residues for SSF led to higher yields of lipase and specific activity. SSF was successfully 10-fold and 17-fold scaled-up to tray-type and pressurized bioreactor,

respectively, with a slight decrease (from 23% to 40%) in total lipase production obtained in flasks. However, no significant differences in specific activity of lipase were found in flasks and in the bioreactors with forced aeration (packed-bed and pressurized). In bioreactors as packed-bed and tray-type, further studies to control moisture content, temperature of the residue and aeration rate should be developed in order to optimize the these variables to improve lipase production.

ACKNOWLEDGEMENTS

Felisbela Oliveira acknowledges the financial support from Fundação para a Ciência e Tecnologia (FCT) of Portugal through grant SFRH/BD/87953/2012. The authors thank the Project "BioInd – Biotechnology and Bioengineering for improved Industrial and Agro-Food processes, REF. NORTE-07-0124-FEDER-000028" Co-funded by the Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER. The authors thank the FCT Strategic Project PEst-OE/EQB/LA0023/2013. Noelia Pérez-Rodríguez acknowledges the financial support of FPU from Spanish Ministry of Education, Culture and Sports. The authors thank to the Spanish Ministry of Science and Innovation for the financial support of this work (project CTQ2011-28967), which has partial financial support from the FEDER funds of the European Union.

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