Production of lipases by solid state fermentation using vegetable oil-refining wastes

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Abstract

Lipases were produced by a microbial consortium derived from a mixture of wastewater sludge and solid industrial wastes rich in fats at thermophilic conditions (temperature higher than 45ºC for 20 days) in 4.5-L reactors and extracted from the solid medium using an extraction buffer (Tris-HCl 100 mM, pH 8.0) and a cationic surfactant agent (cetyltrimethylammonium chloride). Different doses of surfactant and buffer were tested according to a full factorial experimental design. The extracted lipases were most active at 61-65ºC and at pH 7.7 to 9. For the solid samples, the lipolytic activity reached up to 120,000 UA/g of dry matter. These values are considerably higher than those previously reported in literature for solid-state fermentation and highlight the possibility to work with the solid wastes as effective biocatalysts.

Keywords

Solid-state fermentation; Lipase; Thermostability; Organic wastes; Sewage sludge.

1. Introduction

Solid-state fermentation (SSF) is defined as the fermentation process on moist solid substrate in the absence or near absence of free water (Pandey, 2003). SSF can be used for the production of enzymes utilizing various substrates including solid wastes. Lipase production by SSF under different process conditions, with different microorganisms and substrates has been reported (Godoy et al., 2009; Hernández-Rodríguez et al., 2009; Sun et al., 2009). However, most studies were carried out using a few grams of substrate, mesophilic temperatures and pure cultures of known microorganisms. Only a few studies have been carried out at pilot or industrial scales (Kumar et al., 2009; Edwinoliver et al., 2010). SSF encounters problems related to mass and heat transfer phenomena associated with solid substrates (Pandey et al., 2008), and the use of natural solid substrates can hinder downstream processes (Rodríguez-Couto and Sanromán, 2006), especially when extracting lipophilic enzymes such as lipases (Mala et al., 2007). Fermented solids have been used as naturally immobilized biocatalysts for synthesis reactions in lyophilized (Hernández-Rodríguez et al., 2009) or dried form (Hellner et al., 2010). This approach can lead to lower costs of enzyme preparations since no extraction and purification steps are carried out.

The main objective of the current study was to develop a scalable SSF process for lipase production simulating real adiabatic conditions in full-scale processes, to optimize the extraction procedure of the lipases and to evaluate the use of fermented solids as biocatalysts. Waste derived from the vegetable oils refining industry was used as substrate and sewage sludge served as source of microorganisms. The thermostability of the enzyme extract was proved to be effective in commercial tests used for determining the hydrolysis activity and the optimal conditions for extraction were obtained using a full factorial experimental design. Moist and air-dried fermented solids were tested as biocatalysts for the hydrolysis of olive oil, selected as standard hydrolysis reaction.

2. Material and methods

2.1. SSF materials

A mixture of winterization residue (WR) and raw sludge was used as solid matrix for SSF experiments. The main characteristics of the materials are summarized in Table 1. WR was provided by the LIPSA (Lípidos Santiga S.A, Barcelona) oil-refining facility. WR is obtained by submitting vegetable oil to rapid cooling to 5°C over 24 h and removal of waxes by filtering with diatomaceous earth. This waste was selected as a source of fats because of its stable and homogeneous composition according to the manufacturer's information.

Raw sludge (RS) was added to WR as inoculum and co-substrate to provide moisture and nutrients (Gea et al., 2007). RS was collected after centrifugation from the Metrofang wastewater treatment plant of Besòs (Barcelona, Spain), a very big facility treating wastewater of 1.5 millions inhabitants. The RS/WR mixture had 20% of total fats (dry basis) (Gea et al., 2007). Wood chips were added as a bulking agent to the mixture of RS and WR at a ratio of 1:1 (v:v) to provide proper porosity to maintain aerobic conditions. The water content of the mixture was adjusted to 50% by adding tap water before SSF (Table 1).

2.2. SSF experiments

Experiments were undertaken in 4.5 L Dewar® vessels (Sayara et al., 2010) containing 2.5 kg of mixture. The vessels set-up allowed a continuous air supply, temperature monitoring, separate leachate collection and oxygen monitoring to ensure aerobic conditions (oxygen content around 10-12%). Due to the thermal isolation of the vessels, these reactors work under adiabatic conditions, to simulate real SSF processes where a non-constant temperature evolution is produced due to the limitations of heat transfer in organic matrices (Barrena et al., 2006). The SSF experiments were undertaken for 35 days, and samples were collected after 6, 14, 27 and 35 days. The two replicate reactors were opened and the content was mixed well to obtain homogenous and representative samples.

2.3. Enzyme extraction optimization

The entire mass of the SSF reactor was homogenized and 150 g were sampled for extraction experiments. Two grams of this sample were used for each condition proposed in the experimental design. The sample was transferred to an Erlenmeyer flask and supplemented with buffer Tris-HCl (100 mM, pH 8.0) and a surfactant agent. Different doses of surfactant and buffer were tested according to Section 2.4. Cetyltrimethylammonium chloride solution (Aldrich) was selected as surfactant after screening and comparison with other different anionic, cationic and non ionic surfactants (data not shown).

The extraction was carried out at 37ºC on an orbital shaker (100 rpm, 30 min). After 30 minutes the whole content of Erlenmeyer flask was centrifuged at 10000 rpm for 5 min (4ºC) and the supernatant was filtered and used as the enzyme source for the estimation of lipase activity. Prior to the extraction, sample was washed with Tris-HCl buffer (15 mL/g) without surfactant agent to remove soluble compounds (no lipolytic activity was detected).

For the determination of the best conditions for lipase extraction a full factorial experimental design consisting of 15 experiments (12 experiments and three replications at central point for statistical validation) was carried out with the four samples obtained from the SSF process. The selected doses of buffer to wet solid substrate ratio were 15, 90, 200 and 500 mL/g and the surfactant percentages in aqueous extract were 2%, 6% and 10% (v/v). The fraction of lipolytic activity obtained in the extract over the total extractable activity was selected as the objective function. Total extractable activity was estimated by successive extractions from a solid sample until no activity was detected.

The optimization of the proposed polynomial function to obtain the corresponding optimal conditions for extraction was solved by using the Excel solver tool. Statistical testing of model was done by the Fisher’s statistical test for analysis of variance (Anova).

2.4. Lipolytic activity

Lipase activity in solid samples (wet and air-dried) was determined as described by Hernández-Rodríguez et al. (2009), whereas the activity in liquid extracts was measured using a lipase colorimetric assay (kit 1821792, Roche diagnostics, Basel, Switzerland) (Resina et al., 2004). For both methods, one lipolytic activity unit (AU) was defined as the amount of lipase necessary to hydrolyze 1 µmol of ester bond per minute under assay conditions (temperature 30ºC, pH 8) and it was referred to the amount of solid substrate used for obtaining the extract sample, both wet (AU/g) or dry (AU/g DM).

2.5. Effect of pH and temperature on lipolytic activity

The effects of pH and temperature (T) on lipolytic activity were analyzed by a full factorial experimental design consisting of 15 experiments (12 experiments and three replications at central point for statistical validation). The temperatures were fixed at 30, 45, 60 and 75 ºC and the pH at 5.0, 7.0 and 9.0. Residual lipolytic activity (RA, referred to the initial activity of the extracts) after one hour of incubation was selected as the objective function and as a measure of lipase stability. Buffers used for the incubation at the selected pH were: Tris-HCl 1M, pH 9.0; Tris-HCl 1M, pH 7.0; acetic acid-sodium acetate 1M, pH 5.0.

2.6. Analytical methods

Moisture content and organic matter were determined according the standard procedures (Gea et al., 2007). The fat content was measured using a standard Soxhlet method with n-heptane as organic solvent (The U.S. Environmental Protection Agency, Method 9071B).

3. Results and discussion

3.1. SSF experiments

The fermentation process was under thermophilic conditions for 20 days, whereas the evolution of fat content is presented in Table 2. An important reduction in fat content was observed in the first 15 days but no significant fat degradation occurred after that moment, resulting in a final fat content around 5%, which may correspond to fats difficult to biodegrade (Gea et al., 2007).

3.2. Enzyme extraction and lipolytic activity in extracts

Extraction results obtained with the different conditions specified in the experimental design were fitted to different mathematical models. A lineal polynomial expression was used to start and quadratic and interaction terms were added by observing the evolution of the regression coefficient to reach a value where the differences in the goodness of fit were minimal (López et al., 2004). For the four samples analyzed, the best fitting obtained from the experimental design was a full second-order polynomial function according to equations 1-4:

$$Y_6 = 0.0217 + 0.0181x_1 – 0.0063x_2 – 0.0004x_1^2 – 0.0016x_2^2 – 0.0059x_1x_2 \quad (1)$$

$$Y_{14} = 0.7398 + 0.6284x_1 – 0.2027x_2 – 0.0340x_1^2 – 0.0522x_2^2 – 0.1808x_1x_2 \quad (2)$$

$$Y_{27} = 0.0317 + 0.0293x_1 – 0.0090x_2 + 0.0007x_1^2 – 0.0016x_2^2 – 0.0085x_1x_2 \quad (3)$$

$$Y_{35} = 0.0172 + 0.0159x_1 – 0.0047x_2 + 0.0004x_1^2 – 0.0010x_2^2 – 0.0043x_1x_2 \quad (4)$$

where: Y represents the fraction of lipolytic activity obtained over total extractable activity (objective function); x_1 is the buffer dose (mg/L) and x_2 is the percentage of surfactant added (%). Both x_1 and x_2 were normalized values.

Fig. 1 shows the response surface obtained from the above equations for the four samples considered. From Fig. 1 and the coefficient values found in equations 1-4 it can be concluded that the buffer dose has a positive effect on the yield of enzyme extraction, whereas the opposite effect was observed for the surfactant fraction (sign of x_1 and x_2 polynomial coefficients). The effect of buffer dose on extraction yield was more important than surfactant dose, as indicated by the value of x_1 coefficient, approximately three times higher than that of x_2. The optimal values for extraction were 500 mL/g of buffer dose and 2% of surfactant.

As shown in Table 2, only small percentages of activity were recovered even at optimized conditions. However, it was decided not to extend the range of study because doses higher than 500 mL/g would not be economically viable at industrial scale. Other strategies such as a multistep extraction procedure (Mala et al., 2007) should be considered in the future for the scale-up of the extraction process. Values of extracted activity were around 50 AU/g at optimized conditions, and thus in the lower range of values reported in the literature (Hernández-Rodríguez et al., 2009); however, total extractable lipolytic production was in the upper range and the value for the sample from day 14 was higher than previously reported values. The lipolytic activity showed a maximum at day 14, coinciding with the period of maximum fat degradation (Table 2). After 13 days the activity considerably decreased (day 27), which can be attributed to the complete consumption of biodegradable fats (Gea et al., 2007).

3.3. Lipolytic activity in fermented solids

In view of the low extractable activity, the activity of wet and air-dried solid samples was determined (Table 2). A statistically significant difference in the lipolytic activity observed in wet and air-dried samples was observed except for the sample of day 27. Wet samples obtained at the beginning of the process showed higher activity than wet samples taken at later time points, whereas the opposite was noted for the dry samples.

The activity in the solid samples was higher than that of aqueous extracts. The lipase activity in the solids was very high, and it would be of interest to identify the different enzymes that contributed to this high level of activity. A possible explanation for this difference may be related to the low water activity that most lipases require (Hasan et al., 2009).

The levels of lipolytic activity obtained from fermented solids are the highest reported on SSF to the authors' knowledge, two orders of magnitude over any value published. Although lipase activity units reported on literature are obtained with very diverse methods, and often they are not directly comparable, the findings reported here highlight an extraordinary potential for the use of fermented solids as biocatalyst. Additionally, the comparison with other published works should consider that often the enzyme activity is obtained with a pure strain, while in this work the microbial consortia used probably produced a mixture of different lipases with different catalysis potential. The identification of these enzymes could be the subject of future work.

3.4. Characteristics of lipases

The effect of temperature (T) and pH on stability of the extracted enzymes was studied by means of a factorial experimental design. The best fitting for experimental residual activity (RA, referred to the initial activity of each extract, Table 2) that was selected as objective function was obtained for a second-order polynomial model for the four samples analyzed. The equations obtained in this case were:

$$RA_6 = 0.9940 + 0.2700T + 0.0735pH - 0.2357T^2 - 0.0695pH^2 - 0.0339TpH \quad (5)$$

$$RA_{14} = 1.0015 + 0.3408T + 0.0965pH - 0.3299T^2 - 0.0942pH^2 - 0.0554TpH \quad (6)$$

$$RA_{27} = 0.9070 + 0.3063T + 0.0925pH - 0.2709T^2 - 0.0189pH^2 - 0.0518TpH \quad (7)$$

$$RA_{35} = 0.9579 + 0.2078T + 0.0968pH - 0.2657T^2 - 0.0838pH^2 + 0.0192TpH \quad (8)$$

For these equations the regression coefficients (R^2) of $RA_{6,}$ $RA_{14,}$ $RA_{27,}$ and RA_{35} were 0.87, 0.80, 0.86 and 0.77, respectively. In general, it was observed that there was a good correlation, with no statistically significant differences between the estimated and the actual value according to the F-test of the experimental design. Fig. 2 shows the response surface obtained for the equations 5-8. Lipase activity was more sensitive to temperature (T) than pH (the values of coefficients for T are higher than those of pH). The coefficients indicated that high values of both T and pH have a positive effect on residual activity. Lipolytic activity (Fig. 2) was markedly stimulated by temperature in the thermophilic range that indicated a lipase reactivation. There is no clear trend regarding the pH influence on lipase activity, but if this parameter is associated with the temperature, it changes the residual lipolytic activity at alkaline pH with a maximum at pH 9.0. For the four samples analyzed, optimal stability was observed at temperatures in the thermophilic range (61-65ºC) and alkaline pH (7.7-9.0).

4. Conclusions

The study on scalable SSF with vegetable oil refining industry waste has shown that this waste is a good source for lipolytic enzymes production with promising properties. The use of fermented solids as biocatalysts is also promising in terms of low-cost production process with high yield potential. Further research should explore the application of the obtained lipases in novel synthetic routes and their identification. Another point that needs attention is the reproducibility of the source of microorganism used since sludge and, in general, organic solid wastes are inherently variable in chemical composition and in the characterization of the existing microbial communities.

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Figure captions

Fig. 1. Surface response corresponding to lipolytic activity extracted fraction for different doses of buffer and surfactant agent for samples obtained in a) day 6; b) day 14; c) day 27; d) day 35.

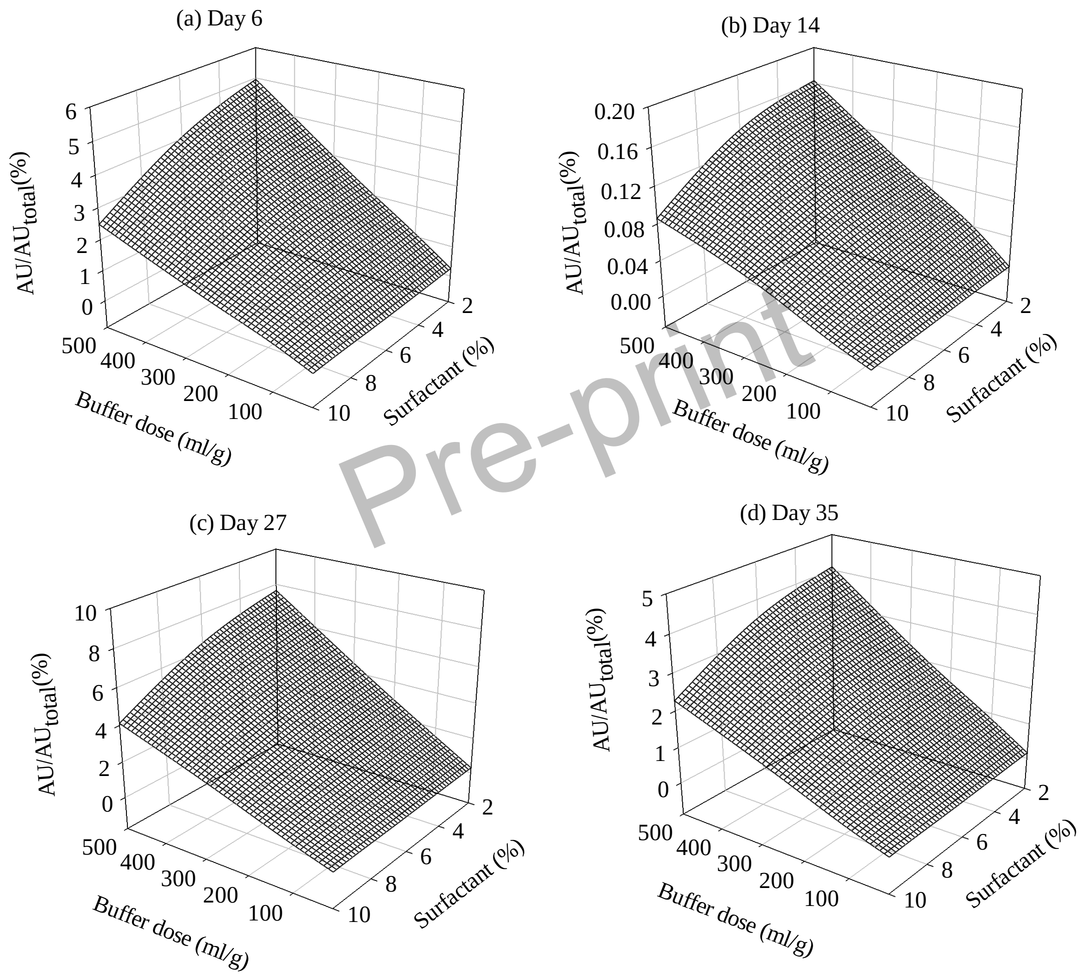
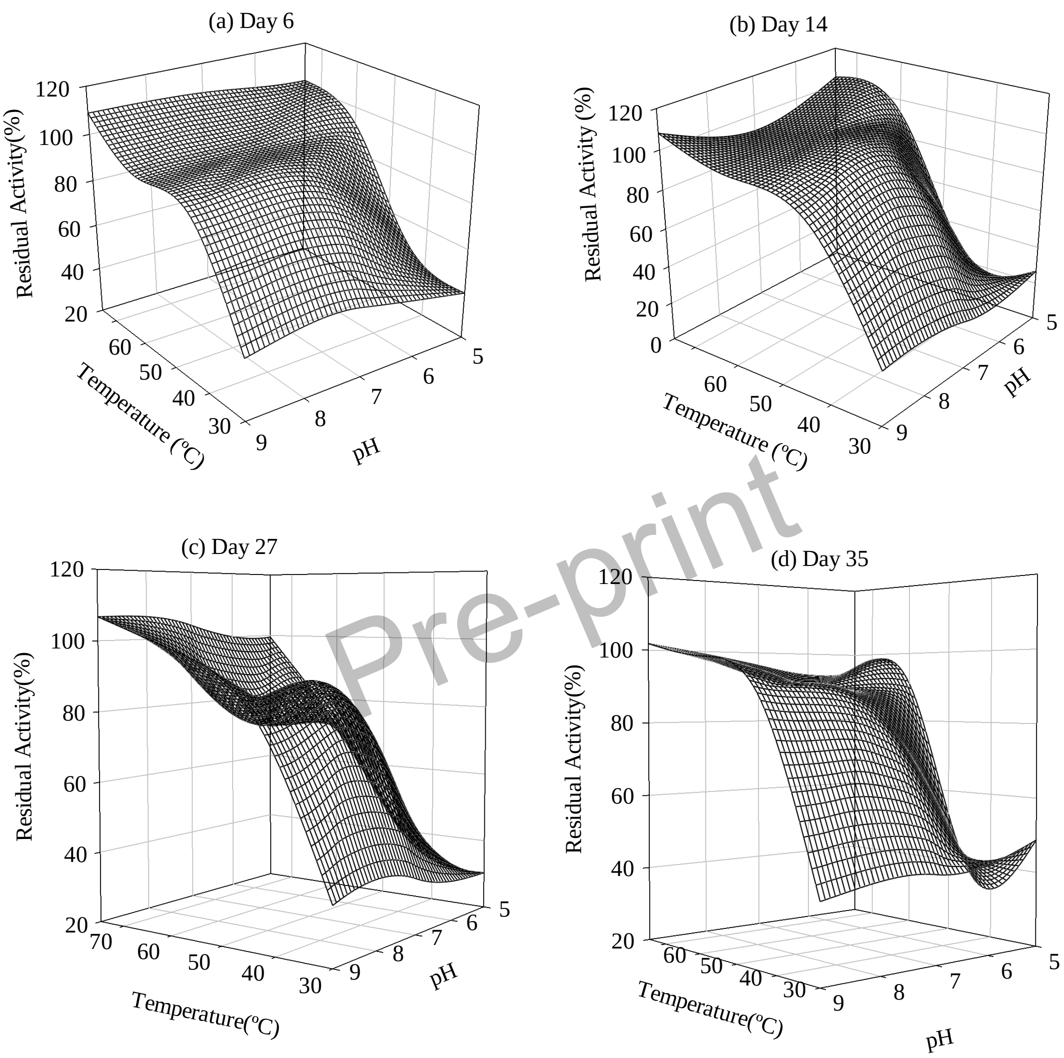


Fig. 2. Surface response corresponding to lipase stability for different pH and temperatures for samples obtained in a) day 6; b) day 14; c) day 27; d) day 35.



Tables

Table 1. Main characteristics of the SSF materials and mixture.

Parameter	Raw sludge	Winterization residue	Wood chips	Fermentation mixture
Moisture (%)	66.9	-	9.1	49.5
Dry matter (%)	33.1	100	90.9	50.1
Organic matter (%, dry basis)	83.5	74.9	-	79.6
Fat content (%, dry basis)	15.9	53.1	-	19.7

Table 2. Lipase activity measured in extracts and fermented solids and total extractable activity.

Sample	Total fat content	Total extractable activity		Enzymatic activity in extract at optimized conditions		Enzymatic activity in solid samples	
	(%, dry basis)	UA_{total}/g	UA_{total}/g DM	UA/g	UA/UA_{total} (%)	Wet samples (UA/g DM)	Air-dried samples (UA/g DM)
Day 6	16	1051	1752	52	5.0	106517	13938
Day 14	5	31550	49113	51.3	0.2	120731	20925
Day 27	6	698	1371	53.4	7.7	87906*	88000*
Day 35	5	1259	2478	51.5	4.1	44928	85251

* Samples with enzymatic activity that is not statistically different.