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Effect of Temperature, Moisture, and Carbon Supplementation on Lipase Production by Solid-State Fermentation of Soy Cake by *Penicillium simplicissimum*

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Abstract

The production of lipases by *Penicillium simplicissimum* using solid-state fermentation and soy cake as substrate was investigated. The effects of temperature, cake moisture, and carbon supplementation on lipase production were studied using a two-level experimental plan. Moisture, pH, and lipase activity were followed during fermentation. Statistical analysis of the results was performed to evaluate the effect of the studied variables on the maximum lipase activity. Incubation temperature was the variable that most affected enzyme activity, showing a negative effect. Moisture and carbon supplementation presented a positive effect on activity. It was possible to obtain lipase activity as high as 21 U/g of dry cake in the studied range of process variables.

Index Entries: Solid-state fermentation; lipase; *Penicillium*, soy cake; olive oil; moisture.

Introduction

The use of lipases in wastewater treatment in the food industry has been proposed to improve process efficiency *(1,2)*. However, economical feasibility depends on low-cost enzyme preparations. Lipase production

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by solid-state fermentation (SSF) may be suitable because this kind of fermentation process presents many advantages over the submerged process, including higher productivity, higher product concentrations, and the use of simpler equipment and low-cost substrate such as agroindustry wastes. In addition, industrial plants for production of enzymes by SSF require low investment and operation costs *(3,4)*.

In a recent study, Castilho et al. *(3)* presented an economical evaluation of lipase production by *Penicillium restrictum* using both submerged fermentation and SSF. They demonstrated that the total investment required for a plant based on submerged fermentation was 78% higher than the investment necessary for installation of an SSF plant. The final enzyme cost in the submerged fermentation process was 68% higher than the cost of a commercial lipase *(3)*. SSF proved to be a highly attractive alternative from an economical point of view. The use of a low-cost raw material (babassu oil extraction waste) coupled with a low investment decreased the final enzyme cost up to 47% lower than the cost of the commercial enzyme *(3)*. The return of the investment was only 1.5 yr and the return rate was 62%, considering a 5-yr lifetime for the project. These aspects, make the production of lipase by SSF extremely promising. In this context, the aim of the present work was to study the effect of some cultivation parameters on lipase production by *Penicillium simplicissimum* using soy cake as substrate for application in wastewater treatment in the food industry.

Materials and Methods

Microorganism

The microorganism used was *P. simplicissimum*, previously isolated by Freire *(5,6)*, using samples of babassu nuts, which were naturally fermented at a babassu oil plant.

Production of Inoculum

The medium used for inoculum production consisted of soluble starch (2% [w/v]), olive oil (1% [w/v]), yeast extract (0.1% [w/v]), $MgSO_4 \cdot 7H_2O$ (0.025% [w/v]), KH_2PO_4 (0.05% [w/v]), $CaCO_3$ (0.5% [w/v]), and agar (1.5% [w/v]). The constituents of the medium were dissolved in deionized water, transferred to a 500-mL Erlenmeyer flask, and then sterilized at 111°C for 30 min. The medium was inoculated with a spore suspension obtained previously from a stock agar slant and incubated at 30°C for 7 d. Spores were collected by adding 10 mL of an aqueous solution of Tween-80 (0.1% vol) and glass beads to the Erlenmeyer flask containing the fermented medium.

Cultivation Medium

The substrate used in all experiments consisted of soy cake, which is the byproduct of soybean oil extraction. The cake was milled and classified (Tyler 35-60) and then stored at –10°C in vacuum packs. Fermentation was

Table 1
Experimental Design and Results of Maximum Lipase Activity

Experiment	Temperature (°C)	Olive oil (%)	Initial moisture (%)	Maximum activity (U/g dry cake)	Time (h)[a]
1	27	0	50	11.1 ± 1.7	64.2
2	27	4	50	16.3 ± 2.4	59.3
3	27	0	70	16.3 ± 2.4	93.4
4	27	4	70	21.5 ± 3.2	94.0
5	33	0	70	9.6 ± 1.4	43.7
6	33	4	70	13.9 ± 2.1	168.0
7	33	0	50	10.9 ± 1.6	85.4
8	33	4	50	10.9 ± 1.6	45.8
9	30	2	60	12.7 ± 1.9	49.9

[a]Time necessary to reach maximum lipase activity.

carried out in 600-mL polypropylene beakers covered with sheets of acrylic fabric. Each experimental point consisted of one beaker with 10 g of cake (dry basis) supplemented with the amount of olive oil and water following a two-level full factorial experimental design (Table 1). The water added with the inoculum was also considered in moisture correction. The beakers with the substrate were sterilized at 111°C for 30 min. The medium was inoculated with the stock spore suspension collected as already described. The final spore concentration in the cake was 10^8 spores/g of dry cake. Spore concentrations were determined using a hematocytometer after appropriate dilution. The beakers were incubated for 7 d in a climatic chamber at different temperatures, with humid air injection. Relative humidity of the air inside the chamber was kept at about 99%. Samples were taken periodically to monitor pH, cake moisture, and lipase activity.

Experimental Procedure

The effect of incubation temperature, initial cake moisture, and olive oil supplementation on lipase production was evaluated following a two-level experimental plan. The range of study of the variables was chosen based on previous results of the our group *(2,7,10)*.

Analytical Methods

Two 0.5-g samples were taken from each beaker for moisture and pH determination. Moisture content was measured by gravimetry, and pH was measured after adding 5 mL of deionized water to the sample, using a pH meter. The enzyme was extracted from the remaining fermented cake by adding 45 mL of sodium phosphate buffer (100 mmol/L, pH 7.0) following incubation at 35°C and 200 rpm for 30 min. After filtration the liquid phase was used for determination of lipase activity.

Lipase activity was determined by adding 2 mL of the extract to an emulsion of olive oil (10% olive oil, 5% Arabic gum in 100 mmol/L of phosphate buffer, pH 7.0.) and incubating at 37°C and 200 rpm for 15 min. The reaction was stopped with the addition of a solution of ethanol and acetone (1:1). The fatty acids released in the hydrolysis were titrated with a solution of 0.04 *N* NaOH *(5)*. Blank tests were performed by titration of a sample of emulsion without incubation, adding the enzyme extract sample just before the beginning of titration. One unit of lipase activity corresponds to the amount of enzyme that produces 1 μmol of fatty acids/min in the reaction conditions just described.

The total nitrogen of the substrate was determined following the standard Kjeldahl method *(8)*. The oil content of the cake was determined by gravimetry after extraction of the samples with *n*-hexane using a Soxhlet extraction apparatus *(8)*.

Results and Discussion

The mean content of oil and grease and of nitrogen of the cake, after milling and classification, was 13.0 and 5.4 wt%, respectively. The C/N ratio of the cake was estimated using the average triglyceride content of the soybean and olive oil *(9)*, considering that the microorganism consumes preferentially the carbon of the oil, which is promptly available in the medium. The estimated C/N ratios for the nonsupplemented and for the 2 and 4 wt% supplemented cake were 1.87, 2.15, and 2.44, respectively. The results show that the cake is rich in oil. This explains the good lipase yields obtained even when the substrate is not supplemented with oil. The mean moisture of the cake was 7.6 wt%. This value was considered for moisture correction of the substrate.

An experimental plan was designed with nine experiments, which were performed in duplicate to estimate experimental errors. The plan is presented in Table 1, as well as the results of maximum lipase activity and necessary fermentation time to reach the maximum activity in each experiment.

It is known that the moisture content in the substrate is essential for good development of the microorganism in SSF *(4)*. Therefore, the moisture of the cake during the fermentation was monitored in all experiments. In most of the studied conditions, the moisture was quite constant with time until 100 h of fermentation, considering an experimental error of 6%. The only exception to this behavior occurred at low initial moisture conditions. In such experiments, there was a strong decrease in moisture of the cake during the course of fermentation. The increase in incubation temperature caused a stronger decrease in cake moisture, probably owing to the higher water uptake rate by the microorganism and the increase in water vapor pressure in the system at higher temperatures.

Figure 1 presents the kinetics of lipase activity for the different studied conditions. In all cases, a maximum in lipase activity could be observed,

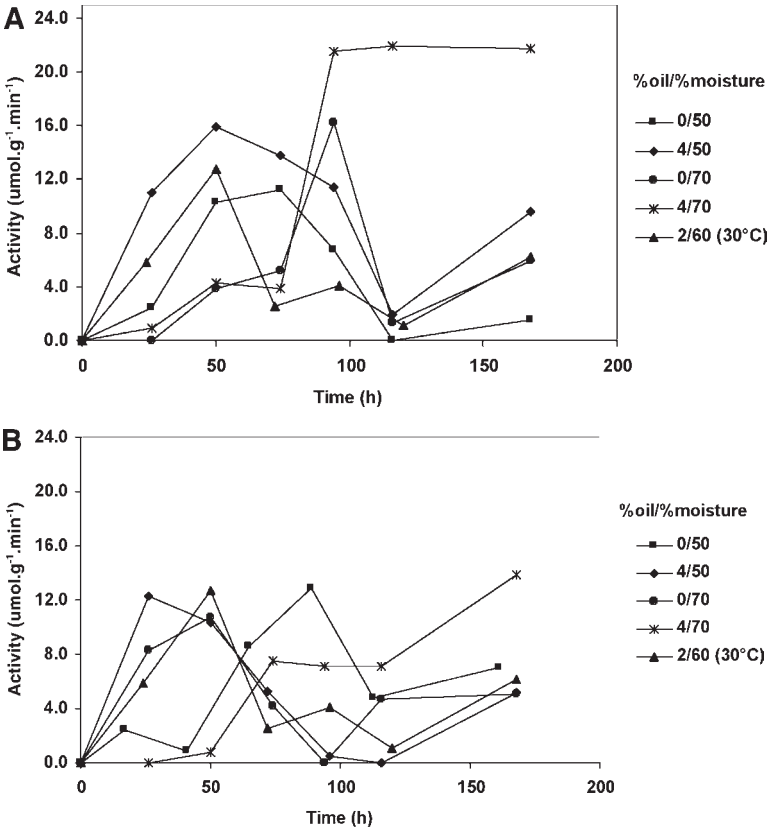

Fig. 1. Lipase production at different conditions of moisture and supplementation: **(A)** 27°C and **(B)** 33°C.

often followed by a new increase by the end of fermentation. The decrease in lipase activity may be related to the effect of pH; temperature; or, still, the protease production by the microorganism, as shown by Gombert et al. *(10)* and Palma et al. *(11)* in previous studies with *P. restrictum*. The presence of proteases in the medium may cause deactivation of the lipase and even the complete loss of lipase activity. The increase in lipase activity at the end of fermentation may be related to the production of a new lipase or decrease in the protease activity. However, new experiments, in which protease activity should be determined, are necessary for confirmation of such a hypothesis. The mean experimental error of lipase activity, calculated based on duplicates, was 14%.

In a preliminary analysis, we noted that the production of lipase was higher at lower temperature (27°C). This result was later confirmed by statistical analysis, which is discussed in the next section. The negative effect of temperature may be related to the increase in the protease activity at higher temperatures.

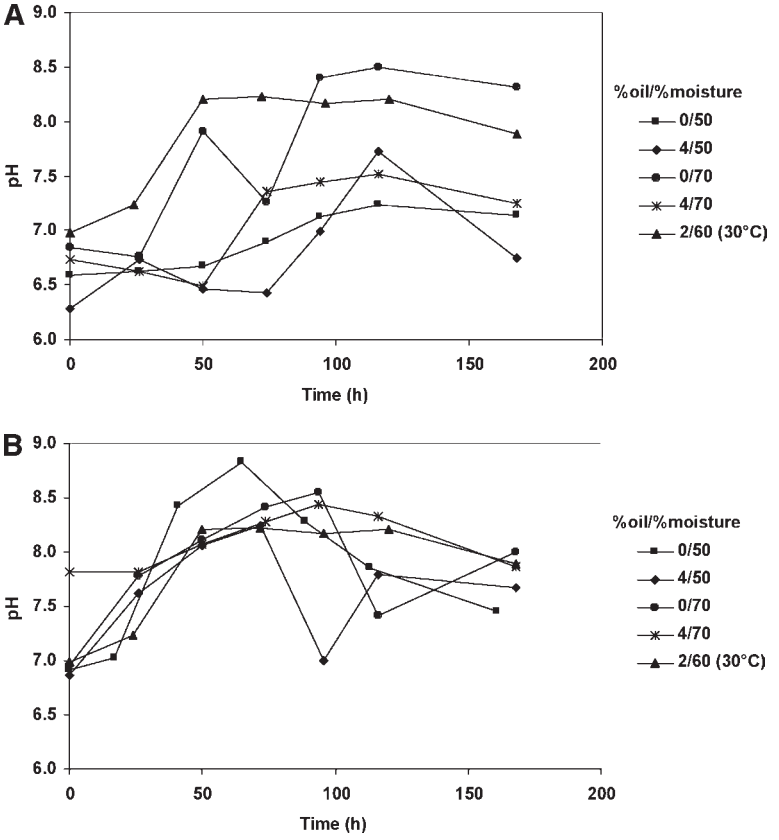

Fig. 2. Evolution of pH during fermentation at different conditions of moisture and supplementation: **(A)** 27°C and **(B)** 33°C.

Supplementation of the substrate with oil led to an increase in lipase activity and a reduction in the time required to reach the maximum activity. This might be owing to acceleration of the metabolism of the microorganism. However, the time to reach the maximum must be analyzed carefully, since factors such as inoculum age and physiologic state may directly affect this result.

Figure 2 shows pH evolution during fermentations. The mean experimental error of pH was 2.2%. pH increased after 24 h of fermentation, and in some experiments it reached 8.5 after 70 h of fermentation. In all experiments the increase in pH may be correlated to the decrease in lipase activity. This behavior is probably owing to the proteolysis, which yields ammonia to the culture medium, increasing pH *(6)*. A decreasing pH trend could also be noted after 100 h of fermentation, matching the new increase in lipase activity. This behavior suggests a reduction in the protease activity in the medium.

Table 2
Best Fit for Maximum Lipase Activity in Function of Temperature, Moisture, and Olive Oil Content [a]

Model: $AT = a_0 + a_1 \times T + a_2 \times OO + a_3 \times M + a_4 \times T \times M + a_5 \times T \times OO$
Dependent variable: AT Loss function: $([OBS\text{-}PRED]^2)/S^2_{experim}$
Final value of loss function: 0.117 R^2: 0.97

Parameter [b]	a_0	a_1	a_2	a_3	a_4	a_5
Estimate	2.76	–0.487	0.371	0.338	–0.256	–0.156
SE	0.07	0.065	0.079	0.076	0.078	0.080

[a] AT, maximum lipase activity; T, temperature; M, moisture; OO, % olive oil; a_0, a_1, a_2, a_3, a_4, and a_5, model parameters; $S^2_{experim}$, experimental variance.
[b] Confidence level >95%.

Statistical Analysis

Statistical analysis of the results was performed using the software Statistica 5.5 (Stat Soft). Maximum lipase activities and time to reach the maximum were calculated through fitting of kinetic curves. The maximum was estimated by derivation of the fits. Empirical models were built to fit maximum lipase activity in the function of incubation temperature (T), moisture of the cake (%M), and supplementation (%OO). The experimental error estimated from the duplicates was considered in the parameter estimation. The choice of the best model to describe the influence of the variables on lipase activity was based on the correlation coefficient (r^2) and on the χ^2 test. The model that best fits the experimental data is presented in Table 2.

Note that the three variables present quite the same effect on maximum lipase activity. Nevertheless, temperature presented a negative effect, which was unexpected, since the metabolism of mesophyllic microorganisms tends to increase with temperature in the range investigated. In this situation, the negative influence might be related to the strong correlation between temperature and substrate moisture. The increase in temperature may lead to the decrease in cake moisture, owing to the increase in vapor pressure of the water. An indication of such an effect may be observed by the strong correlation between moisture and temperature effects (parameter a_4). This correlation also negatively influences the activity to the same degree as the other isolated variables. Other possibilities may be related to a higher level of denaturation of the produced lipase induced by the increase in temperature or to the increase in the proteolytic activity, so that the balance for lipase favors deactivation rather than production.

The concentration of olive oil and moisture content showed positive effects in the range investigated. The increase in olive oil content may favor lipase production by the microorganism owing to accelerated metabolism.

The increase in water content in the medium also increased the metabolic activity of the mold, resulting in a higher lipase production. These results were also observed by Gombert et al. *(10)* for lipase production using *P. restrictum* in babassu cake.

The time to reach the maximum lipase activity did not show any satisfactory correlation with the studied variables. This behavior is probably owing to the high error associated with this variable or with other factors, such as inoculum age and concentration, which were not considered in the statistical analysis and may be influencing this response, although inoculum standardization procedures were used in all the experiments. Fermentation time was not found to be a determinant factor. However, this may be important on an industrial scale, since it can directly compromise enzyme productivity and production costs.

Conclusions

Our study showed that incubation temperature is the variable that most influences lipase production by *P. simplicissimum* using soy cake as substrate, even though it shows a negative effect. To maximize lipase production, fermentation should be carried out at low temperature levels, high moisture in the substrate, and higher concentration of oil. However, good results may also be obtained at lower temperatures without cake supplementation, owing to its high oil content.

The use of soy cake is a promising substrate for lipase production. Even without optimization it is possible to achieve lipase activity as high as 21 U/g of dry cake, comparable with lipase activity obtained with other microorganisms and substrates.

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