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Production of Extracellular Lipase from *Aspergillus niger* by Solid-State Fermentation

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Summary

Lipase production in *Aspergillus niger* J-1 was tested using both submerged fermentation (SmF) and solid-state fermentation (SSF) on a mineral culture medium and wheat bran, respectively. The optimization of the culture medium was carried out for both SmF and SSF. The maximum lipase activity, 1.46 IU/mL, was obtained during the submerged fermentation in a medium containing glucose at 2 % and olive oil at 2 % under conditions of 1 vvm and 450 m^{-1}. However, 9.14 IU/g of dry solid substrate equivalent to 4.8 IU/mL of lipase activity was reached using solid-state fermentation process with a medium containing 0.75 % of ammonium sulphate and 0.34 % of urea. The optimum pH and temperature for enzymatic activity were pH=6 and 40 °C, respectively. The enzyme also exhibited 80 % of its initial activity in neutral and mildly acid media and at temperatures between 20 and 30 °C for a period of 24 hours.

Key words: *Aspergillus niger*, solid-state fermentation, lipases

Introduction

Lipase (E.C. 3.1.1.3) hydrolyses triglycerides to fatty acid and glycerol, and under certain conditions, catalyses the reverse reaction forming glycerides from glycerol and fatty acids. Some lipases are also able to catalyse both transesterification and enantioselective hydrolysis reactions (*1*). The interest in lipase has grown over the last few years due to their excellent catalytic properties (*2*) and their diverse industrial applications, for example, additives in detergents, the elaboration of dietetic foods for use in the food industry, obtaining bioactive molecules in the pharmaceutical industry and pure optical compounds in chemical synthesis processes (*3*), as well as modifications of fats and lipids by hydrolysis and esterification reactions (*4*).

Lipases occur widely in bacteria, yeasts and fungi (*5–7*). Fungi are broadly recognized as one of the best lipase sources and are used widely in the food industry. *Aspergillus niger* is among the most well known lipase producers.

Most of the lipase research focuses on the production of extracellular lipases through a wide variety of microorganisms. Studies on the production of extracellular lipases with *A. niger* have shown variations among different strains. However, the requirement for lipid carbon source remains essential for enzyme production.

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The technique of solid-state fermentation (SSF) involves the growth and metabolism of microorganisms on moist solids without any free flowing water. SSF has many advantages over submerged fermentation (SmF), including an economical use of space that is required for fermentation, simplification of the fermentation media, superior yields and no requirement for complex machinery (*8*). However, SSF has some limitations such as a poor pool of microorganisms capable of growth under restricted conditions and the controlling and monitoring of parameters such as temperature, pH, humidity and air flow (*9*). Although the vast majority of the literature on SSF refers to fungal systems, there are actually very few reports on lipase production in SSF by *A. niger* (*10,11*).

The knowledge and expertise in new microorganisms capable of producing lipase, as well as greater understanding of their properties will be very useful in the application of such systems for some of the purposes mentioned above.

The objective of this study was the production of lipase by *A. niger* by SSF and characterization of the enzyme in regards to its stability in relation to temperature, pH and the optimization of the temperature and pH conditions for obtaining higher lipase activity.

Materials and Methods

Microorganisms and maintenance of culture

Different cultures used in this study were obtained from the microbial culture collection of the Applied Biotechnology Group of the Higher Polytechnic Institute José A. Echeverria. The microorganisms used include: *Aspergillus oryzae* 6.28.1, *Aspergillus niger* J-1, *Aspergillus fumigatus* 6.17.3, *Trichoderma harzianum* 101.8.2, *Mucor griseocyanum* 55.1.1, *Bacillus brevis* 23.9.1, *Bacillus subtilis* 23.44.1, *Bacillus cereus* 23.4.1, *Escherichia coli* 001, *Candida utilis* L 3.75.13, *Candida lipolytica* 3.39.1 and *Saccharomyces cerevisiae* 25.7.3. Potato dextrose agar slants were used for the maintenance of fungal cultures. Fully sporulated slants were stored at 4 °C and subcultured once every three weeks. Nutritive agar slants were used for the maintenance of the bacterium.

Growth media

During the SmF the mineral growth medium (MGM) contained (in g/L): NaH_2PO_4 12, KH_2PO_4 2, $MgSO_4{\cdot}7\,H_2O$ 0.3 and $CaCl_2$ 0.25. Ammonium sulphate at 1 % and olive oil at 2 % were used as nitrogen and carbon sources, respectively. The initial pH was adjusted to 6 for yeast and fungi and to 7 for the bacterium.

The addition of other carbon sources to the *Aspergillus niger* culture was tested. They included: sunflower oil, coconut oil, glycerol, glucose and starch, all at 2 %. It was also assayed with different combinations of olive oil and glucose following the feed scheme represented in Table 1.

Combination 6 was the most suitable carbon source for the *Aspergillus niger* lipase production. It was used to study the effect of air flow and agitation rate on lipase production by way of a factorial design 3×2. The air flow and the agitation rate levels are represented below:

Agitation rate/rpm	Air flow/vvm
X_1	X_2
300 (–1)	0.5 (–1)
450 (0)	1.0 (1)
600 (1)	

The study of the effect of the air flow and the agitation rate was carried out in a Marubishi MD 300-5L fermentor containing 2.5 L of the culture medium.

In the case of SSF, wheat bran was used as a solid substrate and moistened with mineral medium as described above where the salts were added per gram of dry solid substrate (dss). The characteristics of the SSF media are provided in Table 2.

Table 1. Combinations of olive oil and glucose for the production of *A. niger* lipase during SmF

Combination	*w*(glucose)/%	*w*(olive oil)/%
1	2.0	0
2	1.5	0.5
3	1.0	1.0
4	0.5	1.5
5	0	2.0
6	2.0	2.0

Table 2. The composition of culture medium for SSF

	Component	*w*/(%, g/g dss)
Solid substrate	Wheat bran	
Moisture		Variable
Carbon source	Glucose	Variable
Nitrogen source	$(NH_4)_2SO_4$	Variable
	NH_2CONH_2	Variable
Inductor	Olive oil	Variable
Salts	NaH_2PO_4	1.8
	KH_2PO_4	0.3
	$MgSO_4{\cdot}7H_2O$	0.045
	$CaCl_2$	0.0375

The optimization of SSF for the lipase production was carried out by Box-Behnken experimental design. It is a response surface design that only requires three levels for each analyzed parameter. For this research the effect of moisture content, the glucose and olive oil concentrations on lipase production are presented. Table 3 shows the levels used for the three parameters.

Table 3. Levels of moisture, glucose and olive oil concentrations used in the Box-Behnken design to optimize the lipase production from *Aspergillus niger* using SSF

Variable	*w*/(%, g/g dss)		
	Low (–1)	Medium (0)	High (1)
Glucose	1.5	3.0	4.5
Moisture	55	60	65
Olive oil	1.5	3	4.5

At the same time the influence of nitrogen source on lipase production was studied. This variable is not continuous and for this reason it is impossible to include it in the Box-Behnken design. The nitrogen sources employed in each experimental run of the design were: ammonium sulphate at 1.5 % (g/g dss), urea at 0.68 % (g/g dss) and the combination of ammonium sulphate and urea at 0.75 and 0.34 % (g/g dss), respectively.

For the development of the optimization study 17 experimental conditions were analyzed for each nitrogen source. This meant that 51 experimental runs were carried out in triplicate resulting in 153 experimental runs in total.

Lipase production in SmF

The culture was grown in 100-mL Erlenmeyer flasks containing 20 mL of mineral medium. The contents were sterilized by autoclaving at 121 °C for 15 min. After cooling, the sterilized medium was inoculated with spores (10^7/mL) from a 7-day-old culture. The flasks were incubated at 30 °C in a rotating shaker at 100 rpm for 8 days.

For yeast and bacterium 500-mL Erlenmeyer flasks containing 180 mL of medium were inoculated with 20 mL of inoculum and incubated at 30 °C and at 120 rpm for 12 h, and at 37 °C and at 120 rpm for 48 h, respectively.

The mycelium was harvested by filtration under vacuum and later centrifuged at 12 000 rpm for 5 min. The clarified supernatant was used as a source of extracellular enzyme. Culture samples of yeast and bacterium were separated by centrifugation at 12 000 rpm for 5 min.

Lipase production in SSF

A mass of 5 g of wheat bran was taken in 250-mL Erlenmeyer flasks and moistened with 5 mL of sterilized salt solution of MGM (121 °C for 30 min). After cooling, the flasks were inoculated with a spore suspension containing 10^7 spores/mL from a 7-day-old culture grown on MGM and incubated at 30 °C for 7 days.

After one week, 100 mL of distilled water were added to each flask and the mixture was shaken for 30 min at room temperature to facilitate the extraction of enzyme from fermented wheat bran. At the end of the extraction, the suspension was squeezed through a double-layered muslin cloth and it was centrifuged at 12 000 rpm for 5 min. The clear supernatant obtained was used as the extracellular enzyme.

Lipase assay

Lipase production was carried out by measuring the increase in the absorbance at 348 nm due to the release of *p*-nitrophenol during the hydrolysis of 0.4 mM *p*-nitrophenyl propionate at pH=7 and 37 °C. To initialize the reaction, 0.1 mL of lipase solution was added to 2.5 mL of substrate solution (*12*).

Enzyme characterization

Effect of temperature

To determine the effect of temperature on lipase activity, the reaction was carried out at different temperatures ranging from 20 to 90 °C. Crude enzyme and substrate were incubated at various reaction temperatures before starting the experiment and the enzyme assay was performed as described earlier to determine the optimal incubation temperature.

Effect of pH

To study the effect of pH, the lipase activity was measured at various pH ranging from 4 to 10. The pH of the reaction mixture was varied using different buffers (citrate buffer for pH=3–6, phosphate buffer for pH=7–8 and borate buffer for pH=9–10).

pH stability and thermostability of enzyme extract

For thermostability, the enzymatic extract was incubated at 20, 30, 40, 50 and 60 °C for a period of 24 hours. After incubation, the enzyme was immediately cooled in ice bath for 15 min and the residual activity was determined. For pH stability, the enzymatic extract was incubated in a buffer solution at different pH values, ranging from 4 to 10 at 30 °C for 24 hours. After that, the residual activity was assayed.

Results and Discussion

Study of the production of lipase in SmF

The production of lipase by the test microorganisms showed appreciable differences in their lipolytic activity. The mean lipolytic activity reached by fungi was about 5.4 and 2.8 times higher than by the other microorganisms. It could be assumed that the fungal strain was able to efficiently use olive oil both for synthesis of the enzyme and to produce biomass, while the test bacterium and yeast did not demonstrate neither high levels of lipase activity nor high biomass production. However, there is another possibility that both bacterium and yeast strains could be expressing intracellular lipase instead of extracellular mechanism. In a preliminary study for bacterium strain we were able to demonstrate that the intracellular lipase activity of the bacterium was higher than extracellular (data not shown).

Among the strains tested, *A. niger* strain showed more lipase activity. Growth and the enzyme activity of *A. niger* on different substrates are shown in Table 4.

The production of lipase was more significant in culture medium added with lipids as the carbon source than in the culture medium without lipids. It was demonstrated that the lipase activity is induced by the presence of lipid substrates in the medium. Extracellular lipase production by different microorganisms on lipids has been extensively reported (*11*).

The synthesis of the enzyme began around the second day of fermentation and the maximal activity was recorded after reaching the log phase of growth. This suggested that the production of lipase by *A. niger* was partially associated with the growth.

On the contrary, many researchers have reported the positive effect of sugars on lipase production (*13–15*). However, in terms of this study, sugar substrates only favour the growth of the microorganism but not the synthesis of lipase by *A. niger*.

Table 4. Production of extracellular lipase by *A. niger* on different carbon sources

Carbon source	*m*(dry biomass)[a]	Lipase activity[b]
	mg/mL	IU/mL
Olive oil (2 %)	15.2	0.53
Sunflower oil (2 %)	13.8	0.12
Coconut oil (2 %)	13.1	0.17
Glycerol (2 %)	9.32	0.01
Glucose (2 %)	7.72	0.04
Starch (2 %)	3.07	0.03
Olive oil (0 %) + glucose (2 %)	7.72	0.04
Olive oil (0.5 %) + glucose (1.5 %)	9.42	0.32
Olive oil (1.0 %) + glucose (1.0 %)	10.76	0.35
Olive oil (1.5 %) + glucose (0.5 %)	12.82	0.37
Olive oil (2 %) + glucose (0 %)	17.20	0.53
Olive oil (2 %) + glucose (2 %)	23.2	0.99

[a]The biomass was derived from 20 mL of the medium
[b]The culture was grown in SmF as mentioned in Materials and Methods. The activities were measured at the third day of fermentation using *p*NPP as substrate

Different behaviour was observed for the lipase production in the presence of two carbon sources: glucose and olive oil. The best lipase activity, 0.99 IU/mL, was obtained when olive oil and glucose were added to the medium at 2 % (combination 6, Table 1) and the microorganism growth was 23 g/L.

As a result of the optimization of the air flow and agitation rate an enzyme activity of 1.46 IU/mL was obtained under conditions of 1 vvm and 450 m^{-1}.

Study of the production of lipase in SSF

In order to study the effect of moisture, concentration of glucose and olive oil on lipase production in culture medium where ammonium sulphate and urea were used as nitrogen sources, the study was carried out using the Box-Behnken experimental design.

Table 5 shows the maximum values of lipase activity reached with each nitrogen source employed. It can be observed that the highest value of activity was obtained in the presence of the combination of ammonium sulphate and urea. The optimum conditions of the medium are presented in Table 6.

It is necessary to add 1.9 g of liquid (1.9 mL of liquid) for each gram of dry solid substrate to obtain 65 % of moisture. Therefore, 9.14 IU/g dss of lipase activity is equivalent to 4.8 IU/mL.

Comparing the best results of lipase activity obtained using both SmF and SSF, and expressed in terms of the liquid volume of the culture medium, it can be concluded that lipase activity produced by SSF (4.8 IU/mL) is superior to the reported value for SmF (1.46 IU/mL).

For this reason it can be demonstrated that the lipase obtained using SSF is more concentrated, making it an attractive feature from an economic standpoint. Similar criteria are shared by at least one other research group relating to their work on lipase production by *Penicillium restrictum* using SmF and SSF (*16*).

Table 5. Effect of moisture, glucose and olive oil concentrations on extracellular lipase production by *A. niger* with different nitrogen sources

Nitrogen source	Lipase activity[a]	Parameters		
		Moisture	Glucose	Olive oil
	IU/g dss		%	
$(NH_4)_2SO_4$ (1.5 %)	7.24	65	3.0	4.5
NH_2CONH_2 (0.68 %)	7.67	65	1.5	3.0
$(NH_4)_2SO_4$-NH_2CONH_2 (0.75–0.34 %)	9.14	65	1.5	1.5

[a]The culture was grown in SSF as mentioned in Materials and Methods. The activities were measured at the third day of fermentation using *p*NPP as substrate

Table 6. Optimum conditions of the culture medium for the production of *Aspergillus niger* lipase using SSF

	Nonoptimized medium	Optimized medium
Solid substrate	Wheat bran	Wheat bran
Moisture	60 % (g/g dss)	65 % (g/g dss)
Carbon source	3 % (g/g dss) of glucose	1.5 % (g/g dss) of glucose
Nitrogen source	0.75 % (g/g dss) of $(NH_4)_2SO_4$	0.75 % (g/g dss) of $(NH_4)_2SO_4$
	0.34 % (g/g dss) of NH_2CONH_2	0.34 % (g/g dss) of NH_2CONH_2
Inductor	3 % (g/g dss) of olive oil	1.5 % (g/g dss) of olive oil
Salts	1.8 % (g/g dss) of NaH_2PO_4	1.8 % (g/g dss) of NaH_2PO_4
	0.3 % (g/g dss) of KH_2PO_4	0.3 % (g/g dss) of KH_2PO_4
	0.045 % (g/g dss) of $MgSO_4 \cdot 7H_2O$	0.045 % (g/g dss) of $MgSO_4 \cdot 7H_2O$
	0.0375 % (g/g dss) of $CaCl_2$	0.0375 % (g/g dss) of $CaCl_2$

Characterization of the enzyme

Lipase from *A. niger* was optimally active in a temperature range of 40 to 60 °C (Fig. 1). The activity of lipase decreased drastically at temperatures above 60 °C and was totally lost at 90 °C. Overall, lipases from *A. niger* strains have been reported to be active between 40 and 55 °C (*10,17*), while lipase from *Humicola lanuginosa* was reported to be active at 45 °C (*18*).

Furthermore, it was observed that the enzyme extract was very stable and retained about 80 % or more of its initial activity at 20, 30 and 35 °C for 24 hours, while at 50 °C it retained 60 % of its activity (Fig. 1). Similar results have been reported for other fungal lipases (*19,20*).

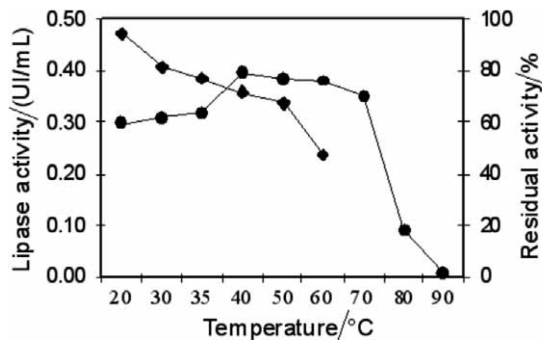

Fig. 1. Activity and stability profiles of *Aspergillus niger* lipase in relation to temperature. Activity (-●-), stability (-◆-)

Enzyme assay was performed under different pH conditions (4–10) and the results are presented in Fig. 2. The optimum lipase activity of the enzyme extract was at pH=6. Similar result was reported earlier by Crueger *et al.* (*21*) for a lipase expressed by another *A. niger* strain.

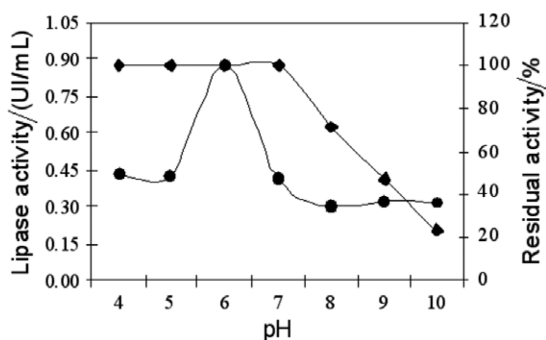

Fig. 2. Activity and stability profiles of *Aspergillus niger* at different pH levels. Activity (-●-), stability (-◆-)

The stability of lipase at various pH levels is shown in Fig. 2. Lipase was very stable in the pH range of 4 to 7 for 24 hours and it retained almost 100 % of its initial activity. However, at pH=8 and 9 the enzyme lost about 40 and 60 %, respectively, of its initial activity in about 2 hours. Most microbial lipases are stable in the pH range of 2 to 10.5, as reported by many of the researchers cited above. Similar results have been reported for other fungal lipases (*20,22*).

Conclusions

In relation to the different test strains, *A. niger* was the best producer of extracellular lipase and its synthesis is induced by the presence of a lipid source. Moderate enzyme activity (9.14 IU/g dss) was obtained in SSF using wheat bran, which to the best of the author's knowledge are amongst the highest extracellular lipase activities reported in the literature concerning fungal sources. This lipase has some properties in common with lipases from other *A. niger* strains. This enzyme is also stable over a broad pH range, 4 to 7, for a period of 24 hours at 30 °C.

Due to the fact that these microorganisms are generally recognized as safe (GRAS) for food, brewing and pharmaceutical applications, more research is necessary to optimize the fermentative process in order to obtain higher lipase production through this strain.

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