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Surface adhesion fermentation for lipase production by *Mucor griseocyanus*

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ABSTRACT

The lipase production by *Mucor griseocyanus* was evaluated using surface adhesion fermentation. Plastic particles, covered with fungal biomass, were produced in the first experimental step. Erlenmeyer flasks (250 ml) were used with whey as culture medium, polystyrene foam as support for fungal growth, and olive oil as inducer for lipase activity. Kinetics were monitored during 72 h of culture time. In a second experimental step, an airlift bioreactor was packed with the plastic particles covered with fungal biofilm and used for production of lipases in batch conditions employing whey supplemented with olive oil. Evaluation of operational conditions indicated that the maximum level of activity was obtained at 60 C and at pH 6.0. It was demonstrated that the fungus grown by adhesion on plastic particles produced the highest activity level (133 U L^{-1}) at 60 h, however, fungal biofilms obtained at 72 h of surface adhesion fermentation had a lipolytic activity at 94 U L^{-1}. For this reason, under these culture conditions, the fungal particles were produced and then packed into the airlift bioreactor where the lipase activity was enhanced. Two sequential batches were evaluated using the same particles of polystyrene foam covered by fungal biomass. The fungal covered particles can be used and reused to produce lipases.

Key words: Airlift bioreactor, lipases, *Mucor griseocyanus*, surface adhesion fermentation.

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INTRODUCTION

Lipase (E.C. 3.1.1.3) acts by hydrolyzing triglycerides to fatty acids 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 optical isomer-specific hydrolysis reactions[4].

These hydrolytic enzymes are widely distributed in nature and are present within the degradative metabolic processes of some microorganisms, plants and animals. However, the microorganisms are the primary commercial source of these enzymes. After microbial production, recovery and purification, the enzymes can be used in free and immobilized forms. Enzymatic immobilization has many advantages, because it focuses on operational benefits of the process, including the reduction of biocatalysts cost, the ability to develop continuous bioprocesses (enzymes reuse) and higher productivity.

Lipases are produced by many microorganisms. Typical examples are lipases produced by *Aspergillus niger* and *A. fumigatus*[3]. Recently lipase and penicillin acylase were reported from *Mucor griseocyanus*[1,2,6]. These enzymes are commonly produced by various processes, such as solid-state or submerged fermentations. However, the benefits of other systems, such as "surface adhesion fermentation," a new fermentation category, can be applied as a method for fungi enzyme production[11]. There have not been many reports about surface adhesion fermentation (SAF). This technique uses fungal adhesion to synthetic or natural surfaces. In the process, spores are adhered to the solid support (natural or synthetic), and the cells grow evenly to form a biofilm. Natural adsorption on solid supports is an immobilization technique that has been proven on filamentous fungi[11].

In this study, recycled polystyrene particles were used as support for *Mucor griseocyanus* biofilm to produce lipases in batch systems of flasks and an airlift bioreactor using whey supplemented with olive oil.

MATERIALS AND METHODS

Microorganism and culture media. A freeze-dried *Mucor griseocyanus* Hagem, strain H/55.1.1, was used. The fungus was provided by the microbial culture collection of the Nanobioscience group, School of Chemistry, Universidad Autónoma de Coahuila. Inoculum was prepared by culturing the spores on potato dextrose agar (PDA) for 7 days at 30 C. The spores were recovered with a 0.01% Tween 80 solution and counted in a Neubauer chamber.

Preparation of support. Previously used polystyrene foam was obtained from the Burblepack company, and it was cut into small irregular pieces of 1 cm^3. Pieces were washed in distilled water three times to clean them thoroughly, and then dried at room temperature (25±2 C). Particles were stored in plastic hermetic bags and were used as support for fungal growth. Porosity of the foam promotes the adhesion of fugal cells.

Surface Adhesion Fermentation (SAF) in Erlenmeyer flasks. First, 0.2 g dried polystyrene portions were weighed. Then the polystyrene foam samples were sterilized using a UV lamp for 24 h, and placed aseptically into 250 ml Erlenmeyer flasks. Next, 30 ml of sterile culture medium were added. Whey at pH 5.5,

previously sterilized in autoclave at 121 C for 8 min, was used as the nutrient source. Each flask was inoculated with 10^7 spores per ml of culture medium. Olive oil at 2% was added as inducer. All flasks were placed on a shaking platform (120 rpm) at 30 C for a period of 72 h. Two flasks were removed from the shaker every 12 h. The fungal growth was measured as dry mass of the mycelium (g) per sample. The support with biofilm was separated from culture medium by filtration on a strainer, and dried at 60 C to constant weight. Free-grown fungal biomass was separated from recovered broth by filtration using a pre-weighed filter paper (Whatman no. 41), and oven-dried at 60 C to constant weight for biomass determination. The cell-free culture broth was centrifuged at 13,000 rpm for 5 min, and assayed for extracellular lipase activity.

Assay for extracellular lipase activity. Lipase activity was assayed spectrophotometrically at 37 C using 0.1 ml of solution containing lipase and 2.5 ml of 0.4 mM *p*-nitrophenyl propionate (*p*NPP) as substrate in 0.05 M phosphate buffer at pH 7. The increase of absorbance at 348 nm, due to the *p*-nitrophenol release, was measured[10]. One unit of activity (U) was defined as the amount of enzyme that hydrolyzed 1 μmol of *p*NPP per min under the described conditions.

Effects of temperature and pH on the enzyme activity. To study the effect of temperature, the lipase activity was measured at various temperatures ranging from 0-80 C. For the optimum pH, activity was measured according to standard assay at pH in the range of 4-9 using 0.05 M acetate (pH 4-5), phosphate (pH 6-8), and borate (pH 9) buffers.

Production of lipases in an airlift bioreactor. In a second experimental step, a 3 L airlift bioreactor (acrylic) was packed with the plastic particles covered by fungal biofilm obtained under selected culture conditions, and used for production of lipases in batch conditions using whey with olive oil.

Experimental design and data analysis. For the first experimental step, three different experimental designs with monofactorial fix were used to evaluate the time of production, optimum pH and temperature of fungal lipases. All kinetic measures were in triplicate and analyzed to obtain the means and standard deviations.

RESULTS AND DISCUSSION

Biomass production. Kinetics of both free and immobilized *Mucor griseocyanus* biomass formation are shown in **Fig. 1**. The production was monitored every 12 h for 72 h. The biomass attached to the polystyrene foam increased gradually until the end of the fermentation. **Fig. 1** shows that during the first 12 h of fermentation, 1.203 g biomass were attached to polystyrene. After each 12 h period, the biomass profile behaved differently. The free biomass was less than the attached biomass. The free biomass decreased every 12 h, until 48 h, followed by increases at 60 h and 72 h. Most biomass was attached to the polystyrene with a final weight of 2.041 g/0.2 g of support.

Lipase and biofilm production. It is known that fungi grown on either natural or synthetic surfaces constitute or form biofilms, and show distinctive physiological characteristics[9]. The biofilms can influence the expression of the enzyme or secondary metabolites of interest. This study showed that whey (a byproduct of the dairy industry) can be used to produce high

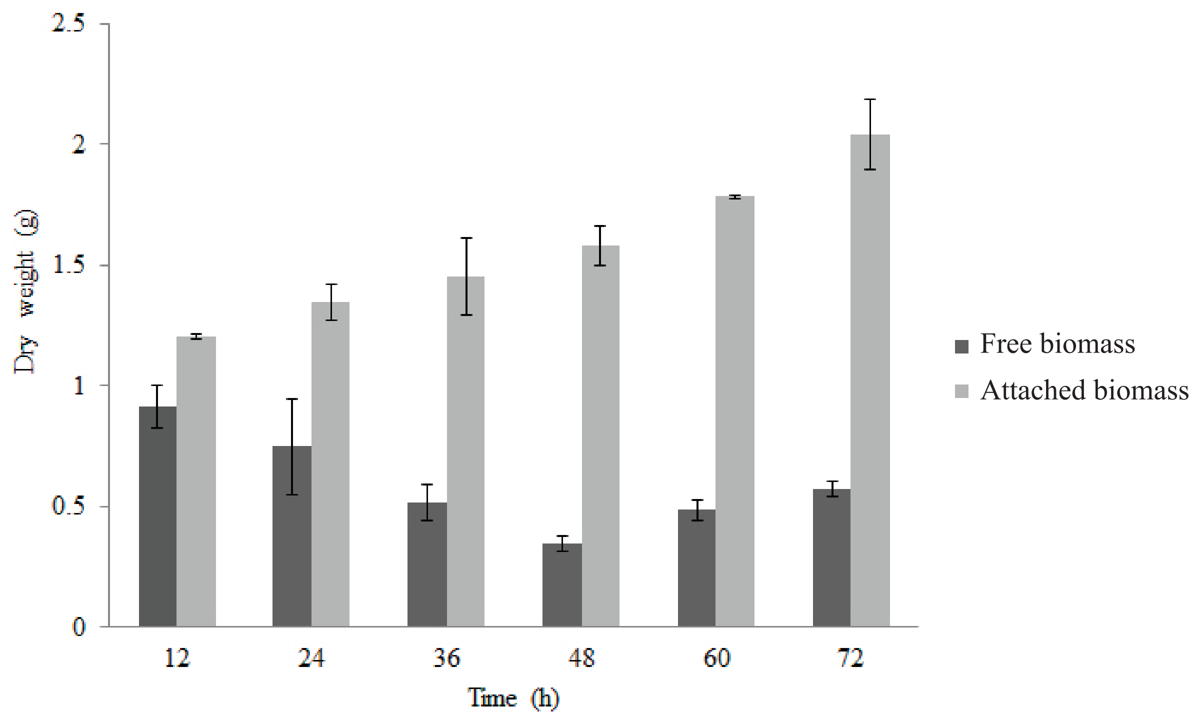

Fig. 1. Dry weight of attached and free biomass of *Mucor griseocyanus* produced during surface adhesion fermentation, using polystyrene as support in whey.

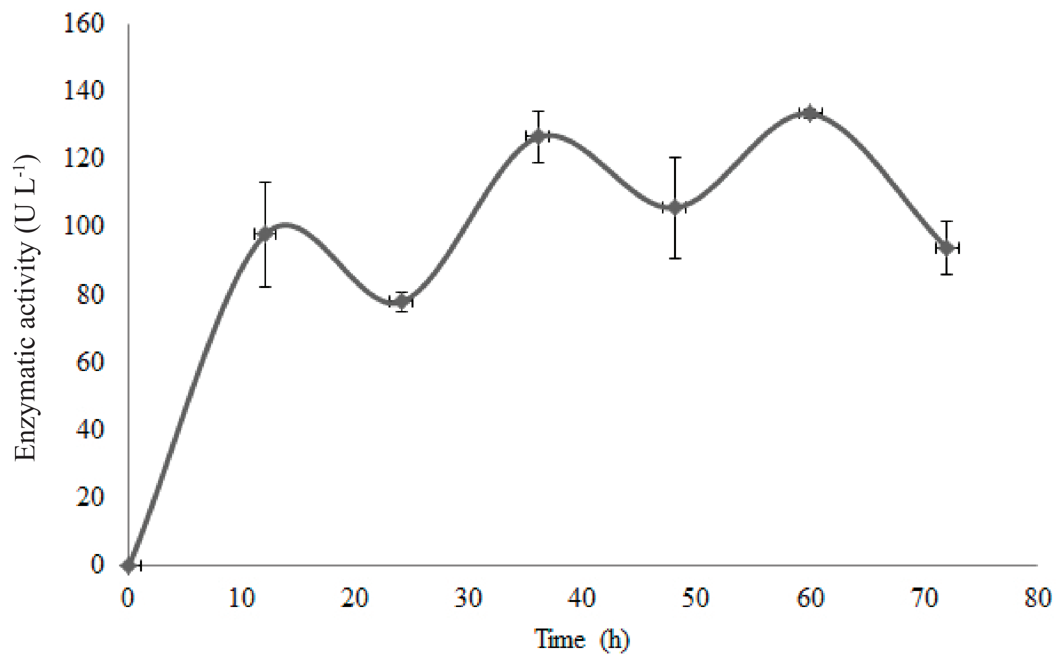

Fig. 2. Lipase production by *Mucor griseocyanus* during surface adhesion fermentation, using polystyrene as support in whey.

yields of lipase by *M. griseocyanus*. Whey provided greater yields than previously reported data.

The objective of this study was to determine if *M. griseocyanus* is capable of producing a resistant biofilm, which would adhere strongly to the substrate and increase the production of the enzyme. **Fig. 2** depicts the lipase production by *M. griseocyanus* in SAF with whey as nutrients source. Extracellular lipase activity was detected after 12 h of fermentation. The activity then increased, reaching an activity of 137 U L^{-1}at 60 h. The activity in the range of 110 to 140 U L^{-1} decreased after 60 h. The decrease could be attributed to the presence of proteases. Whey is rich in proteins, which could induce the production of proteases. It is also possible that the decrease might be due to the limitation of mass transfer inside the mycelial mass. These aspects have been discussed[8], and observed in a similar SAF enzyme production[7].

Enzyme characterization. **Fig. 3** shows that the enzyme's greatest activity occurred between 40 C and 60 C. Greater temperatures showed a sharp decline in activity, probably due to thermal inactivation of the enzyme. It has been reported that most lipases are active between 20 C and 40 C[3]. The optimum temperature obtained was similar to that for the lipase excreted by *Aspergillus niger*[4].

The lipases produced by *Mucor griseocyanus* were characterized. The influence of temperature and pH were determined giving a defined profile. The influence of temperature was analyzed at a constant pH of 6. The influence of pH was studied at a constant temperature (37 C). The enzyme showed optimal activity at pH 6.0 (**Fig. 4**).

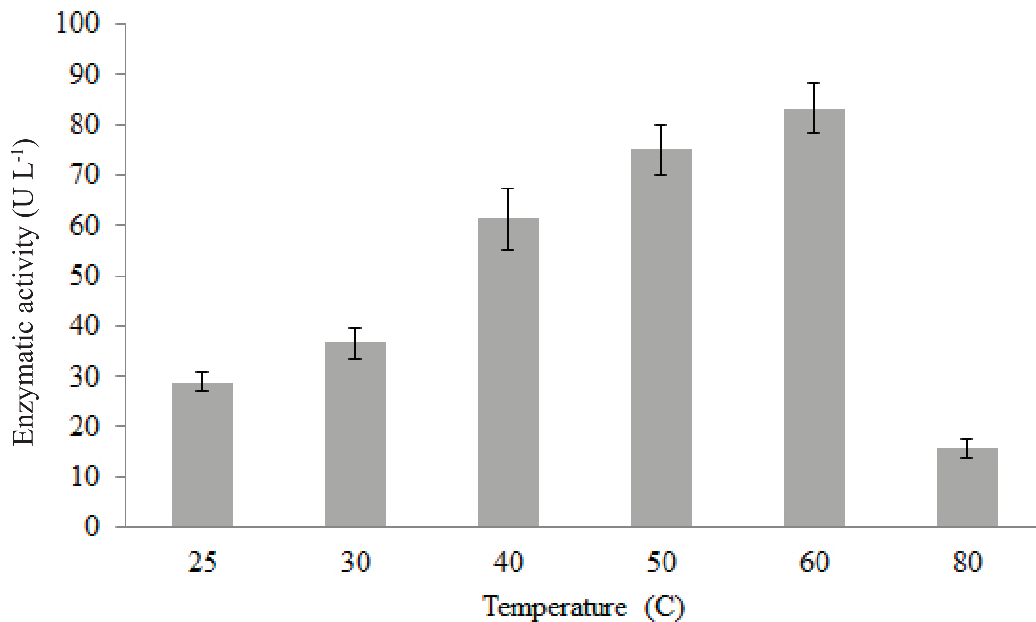

Fig. 3. Effect of temperature on lipase enzyme activity of *Mucor griseocyanus* (crude enzyme extract was obtained at 60 h of surface adhesion fermentation in whey).

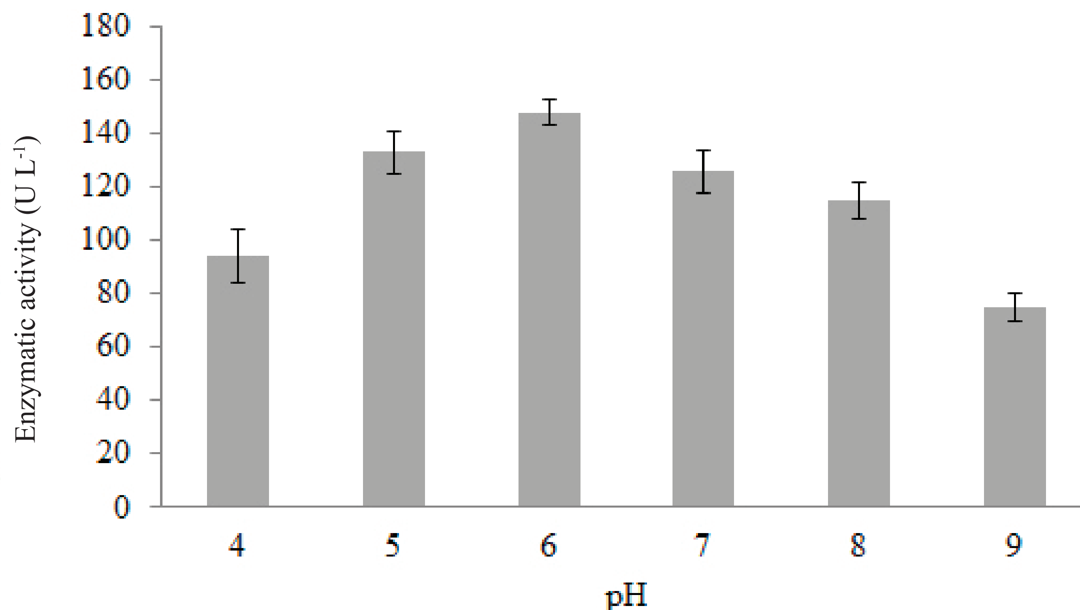

Fig. 4. Effect of pH on the enzymatic activity of lipases of *Mucor griseocyanus* (crude enzyme extract was obtained at 60 h of surface adhesion fermentation in whey).

Lipase production by SAF in airlift bioreactor system. In **Fig. 5**, the first cycle maximum activity, 110 U L^{-1}, occurred at 60 h, while the second cycle, with activity of 140 U L^{-1}, was at 36 h after the replacement of medium. Finally, at 72 h it reached 160 U L^{-1}, about 30% greater than in the first cycle. The SAF seemed to provide a significant advantage. The reuse of biomass permitted greater activity in less time. Filamentous fungi are naturally adapted to growing on surfaces; they demonstrate a physiological behavior, which is different from that of submerged culture[5].

We can conclude that the crude enzyme lipase from *Mucor griseocyanus* differ in some of its properties, and also displayed kinetic properties comparable to those reported for lipases from other fungi. The behavior of this novel enzyme was shown by the activity at high temperatures and for its stability at moderate temperatures.

Fig. 5 shows the enzyme activity inside of a bioreactor with the preformed biofilms; the activity detected from 12 h with 60 U L^{-1}, but at 48 h fell to 30 U L^{-1}. Then, at 60 h, the detected activity was 110 U L^{-1}, representing an increase of about 3 times with respect to that at 48 h. This can be attributed to a phase adaptation of the mycelium to the substrate. In the second repeat, the activity reached 35 U L^{-1} at 12 h; at 24 and 36 h the activity reached 50 and 120 U L^{-1}, respectively. At 48 h the enzymatic activity was 135 U L^{-1}, and again increased to 150 U L^{-1} at 72 h.

There are no reports of fermentations by SAF for production of enzymes, with the advantage of the use of the biomass produced in airlift bioreactor systems. We recorded significant differences during the evaluation of the bioreactor into two cycles.

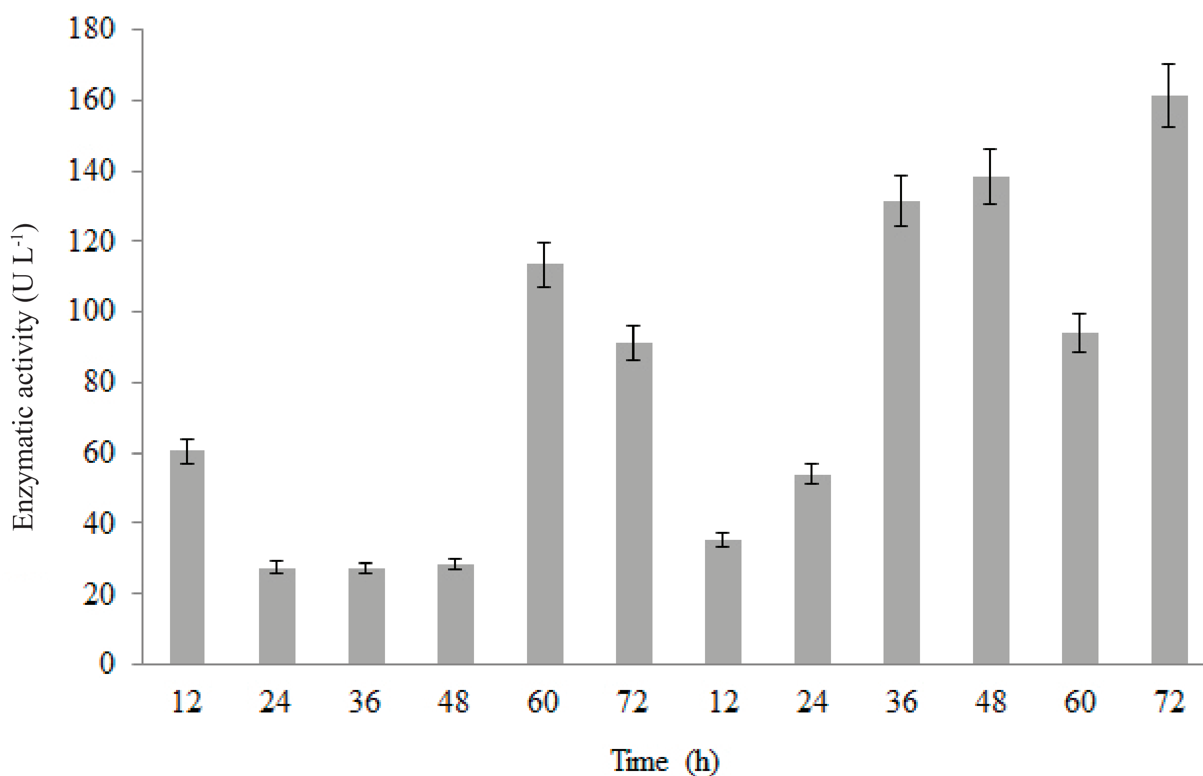

Fig. 5. Lipase production by *Mucor griseocyanus* in a 3 L airlift bioreactor by means of surface adhesion fermentation (SAF), using polystyrene as support and whey as nutrients source (at pH 5.5, at 30 C, and 2 vol air/vol liquid/min).

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