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Effect of Solid-state Fermentation Parameters on Growth of Interest and Environmental Enzyme Production with *Aspergillus niger*

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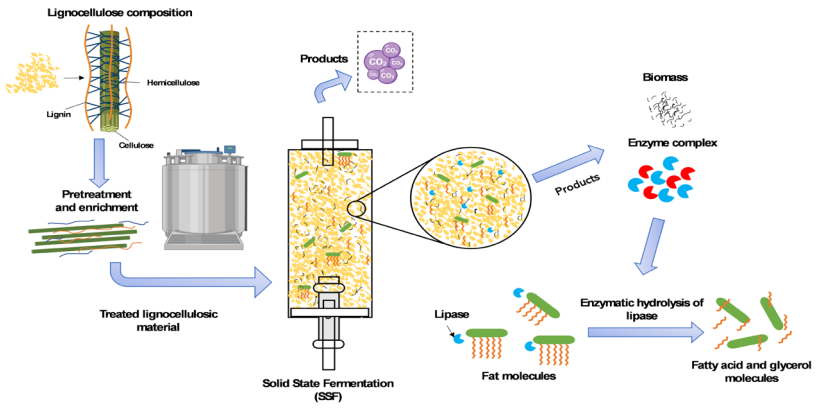
Using residual biomass in biological processes is increasingly promoted to alleviate environmental impacts. However, the focus is mainly based on emerging technologies and limited resources, and the conditions of the technologies remain unclear. The objective of this research is to establish the conditions of a solid-state fermentation system (SSF) for the growth of fungal biomass and the production of ligninolytic enzymes of environmental interest using *Aspergillus niger.* In a screening step, the biomass and lipase enzyme expression of three *A. niger* strains obtained from different screening sites will be extended. In the second stage, the effect of packing density (ρs) and airflow (vvm) was evaluated through a 2^3, on the growth of fungal biomass, fiber degradation, and CO_2 generation in a lipid-contaminated SSF system. The generation of biomass and the concentration of specific enzymatic activity (U/L) of lipase present a correlation for all the strains evaluated. It was estimated that the *A. niger* strain AN19bc isolated from sugarcane bagasse presents the highest accumulation of biomass and concentration of specific enzyme activity of lipase (0.027 U/L) after nine days. The most appropriate conditions for the production of fungal biomass of the AN19bc strain in an SSF system are presented with an airflow of 33.33 vvm and packing density ρs = 360 kg Mss/m^3, levels with which Yx/s= 0.91 is reached. After the process, the solid support used in the SSF presents a change in composition, with the fiber being the component that suffers a considerable degradation of 78.70%.

Graphical abstract



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1. Introduction

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Lipase is a hydrolytic enzyme or also called triacylglycerol lipase, which breaks down triacylglycerols into glycerol, free fatty acids, monoacylglycerol, and diacylglycerol. This enzyme is part of a very important group of biocatalysts used in biotechnological applications in the pharmaceutical industry to obtain pure enantiomers [1], in addition, to the synthesis of polymers for the textile industry [2], formulation of detergents, the cosmetology industry [3], the food industry to reduce and convert lipids, synthesis of esters, among others [4].

Lipase is generally obtained through biosynthesis from plants, animals, and microorganisms [5]. On this, the literature indicates that microorganisms with high lipolytic capacity are isolated from residues from the production of vegetable oils [6], dairy industries [7], soils contaminated with oils or fats, and deteriorated foods [8]. Even though there are countless microorganisms capable of producing the enzyme lipase, molds of the genus *Aspergillus* and *Penicillium* have shown the highest production values of this enzyme in solid and liquid culture media, which represents a potential source for means of biotechnological production [9].

In 2019, the enzyme market had an annual growth of more than 6% [10]. One of the main characteristics of lipase is that it acts on insoluble compounds and aggregates, which is why they operate together at the lipid-water interface. Under these conditions, an increase in the catalytic activity can be produced and this can be decisive concerning the concentration and the conditions used in the processing [11].

The solid-state fermentation system (SSF) allows microorganisms to increase, decrease or remain constant. In SSF systems bioreactors, lignocellulosic residues are generally used as support and substrate so that microorganisms can perform their biological and growth functions [12]. Many microorganisms are capable of producing molecules of interest in this system. Production values of proteins [13], organic acids [14], and enzymes of environmental interest, such as cellulases [15], laccases [16], and lipases [17], have been reported. Thus, research is currently being carried out on the development of remediation bioprocesses [18] using the enzymes produced in SSF systems [19].

The solid support characteristic of SSF systems can affect in many cases the accessibility of the substrate to microorganisms [12]. Parameters such as the porosity and the packing density of the solid support can influence the growth of microorganisms (μmax), as well as, in the mass and heat transfer processes, the elimination of CO_2, and the metabolic heat generated [20]. To obtain success in SSF, it is necessary to consider the relationships that exist between the physiology of microorganisms and physicochemical factors [21]. Therefore, the configuration and monitoring of the SSF parameters are of great importance to achieving optimal development of the process [22].

The objective of this research is to establish the most appropriate parameters for the growth of fungal biomass and subsequent expression of lipase enzymes of environmental interest, in a solid-state fermentation system (SSF) using *Aspergillus niger* on a sugarcane bagasse support. sugar.

2. Results and Discussion

2.1 Selection of the microorganism with lipase production capacity

According to studies carried out by Cihangir and Sarikaya [23], it is important to isolate and select strains with greater lipolytic power. It was determined that all the strains isolated in this study present lipase activity (Table 1). The AN19bc coding strain isolated from sugarcane residues had the highest activity, with an average of 0.021 U/L, followed by the AN19dc strain which was isolated from coffee industry residues with an average of 0.018 U/L.

Ribero et al [24] report maximum lipase activity values of 27.46 and 30.76 U/mL with *A. niger* in submerged fermentation systems, values similar to those found in the present investigation, while Falony et al [25] when reporting enzyme activity values for solid-state fermentations (4.8 U/mL) and liquid fermentation (1.46 U/mL).

Lipase activity is associated with the composition of the substrate used for its growth [5], sugarcane bagasse is rich in glucose, which makes it attractive as a substrate for the microorganism [26], compared to rice husks and coffee waste, which would explain in a certain way the higher level of production of enzymatic activity observed for this strain. This is because its adaptation in the contaminated medium was faster, as demonstrated by Colla et al [27], Zulma et al [28], and Nema et al [29] in their research.

Table 1. Classification of the *A. niger* strain with the highest lipase enzyme production.

Sampled Type	Strain Code	Enzymatic activity (U/L)
Rice husk	AN19ca	0.017 ± 0.003
Coffee wishes	AN19dc	0.018 ± 0.002
Sugarcane bagasse	AN19bc	0.021 ± 0.002

2.2 Lipase enzyme kinetics

Figure 1 shows the kinetics of expression of lipase activity during 15 days of submerged fermentation (SmF), where it is determined that the synthesis of the enzyme begins in the latency stage. At 24 hours, the lipolytic activity of the AN19bc strain was 0.22 U/L, reaching its maximum activity at nine days, with a maximum lipase activity of 0.27 U/L. Rajendran et al [30], and Wong & Farres [31], reported similar results to this investigation, where they determined that lipase activity gradually increases after 8 hours of fermentation.

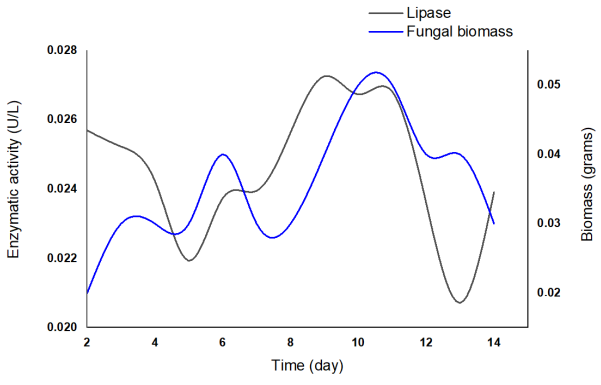


Fig. 1. Kinetics of the enzyme *A. niger* lipase (AN19bc) in SmF.

On the other hand, it was observed (Figure 1) that the production of lipase is accompanied by the generation of biomass of the AN19bc strain of *A. niger*, which reaches its maximum production on day 11 with a value of 0.05 grams. This corroborates the reports made by Terebiznik, who mentions that the biomass production of *Aspergillus* fungi is

associated with enzymatic expression [32], that is, when this fungus grows, the production of enzymes necessarily increases [33]. Regarding this association, it is also mentioned that nutrient saturation induces osmotic changes in the system, reducing the efficiency of the enzymes involved in the transformation of the substrate [34], and causing the values of biomass production to decrease.

2.3 Influence of *ρs* and vvm on the growth of AN19bc in SSF

2.3.1 CO_2 and biomass production

The biomass and CO_2 concentrations, as well as the fiber degradation of the solid support (sugarcane bagasse), are shown in Figure 2. The biomass production had similar behavior in all the evaluated treatments, however, it is observed that the highest concentration of biomass (4.32 g) occurs with an airflow of 33.3 vvm and a density of 360 kg MSS/m^3, a combination of parameters with which the highest coefficient of performance Yx/s (0.91gg^{-1}) is presented, as well as, due to the effect of growth, the degradation of 78% of the fiber of the support is presented.

Studies carried out by Ahamed and Vermette [33] report a biomass production of *Trichoderma reesei* with a value of 2.21 g. Meanwhile, Chuppa et al [35] report values of 0.25 to 0.41 g with *A. niger* on a solid fermentation system. Regarding the yield coefficients, yields of 0.511 and 0.47 g g^{-1} respectively [35, 36] are reported, while others reported values higher than 0.979 g g^{-1} [37]. According to the literature, filamentous fungi contain long and branched hyphae, where increasing the surface area of the substrate improves their interaction and therefore the productivity of the enzyme [38].

CO_2 production is directly related to biomass production, both have a linear evolution, which was also determined by Dustet and Izquierdo [39], where they indicate that the solid medium is adequate to promote the growth and production of enzymes of this fungus [40]. According to Aguilar et al [41] at the time when the highest rate of CO_2 production occurs, initiates the synthesis of metabolites such as enzymes, in addition, studies reported by Thomas et al [42] mention that CO_2 production is linearly correlated with the production of extracellular enzymes such as lipases, as well as with biomass production.

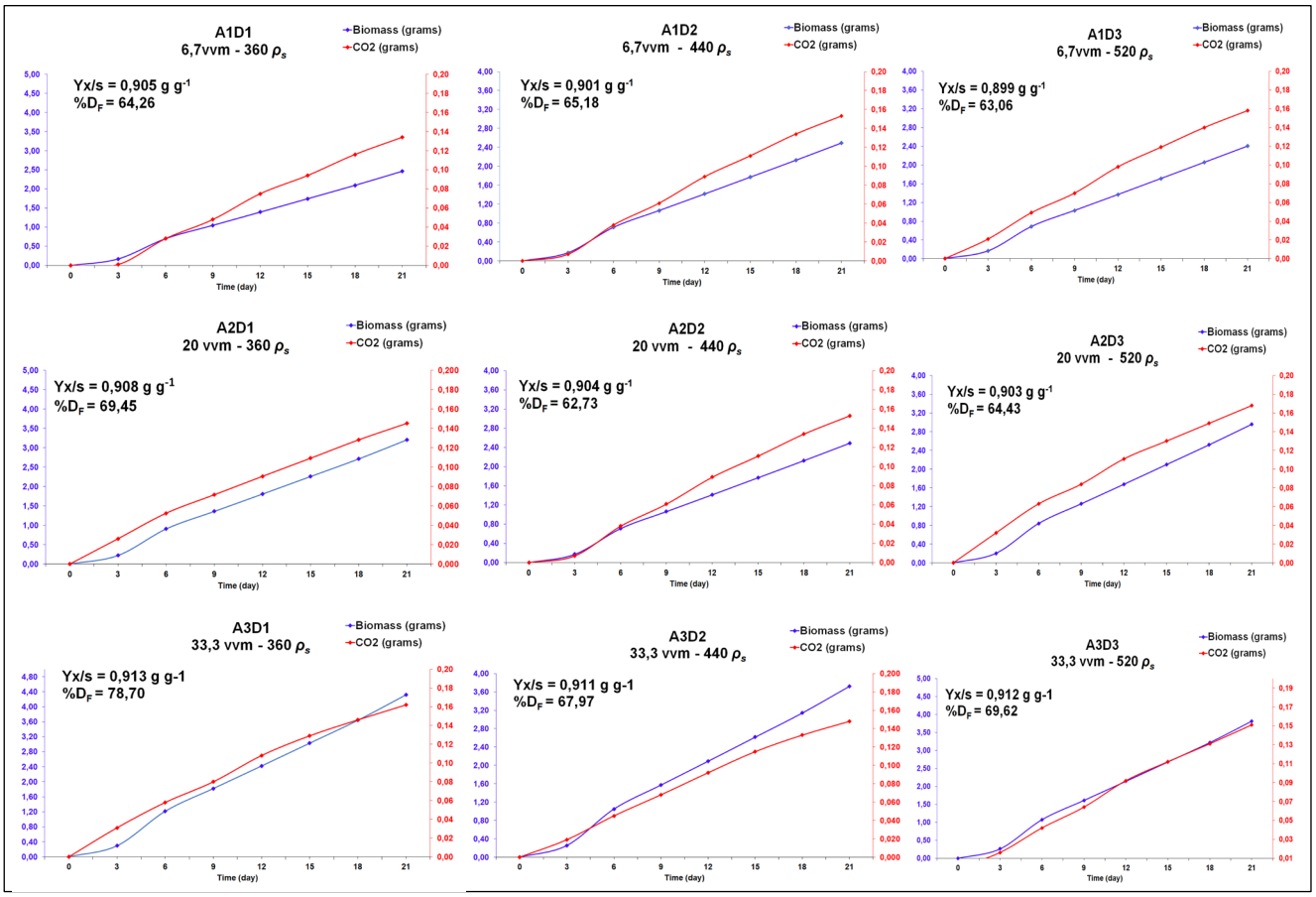


Fig. 2. Production of biomass and CO_2 during the SSF process at the different conditions of packing density and airflow.

Studying the kinetics of biomass and CO_2 production allows for optimizing the factors that affect the production of biomass and enzymes [43]. In the Pareto diagram for biomass production (Figure 3A), it is shown that airflow (vvm) and packing density (ρs) factors exert a significant effect, both with opposite physical sense. Díaz et al [44] and Gutarra et al [45] agree with this criterion since they determined that vvm has a significant effect on the production of biomass and enzyme ellagitanase and lipase in *Aspergillus niger* in SSF.

Regarding the fiber degradation (FD), the vvm and ρs exerted a significant effect, in turn, the highest levels of vvm positively influenced the FD, studies carried out by Mohamad et al [46] and Evans et al [47], demonstrated that the significant decrease in fiber coincides with the production of fungal biomass of *A. niger* and *T. viride* since the carbohydrate content contained in the residue acts as a carbon source for the growth of microorganisms.

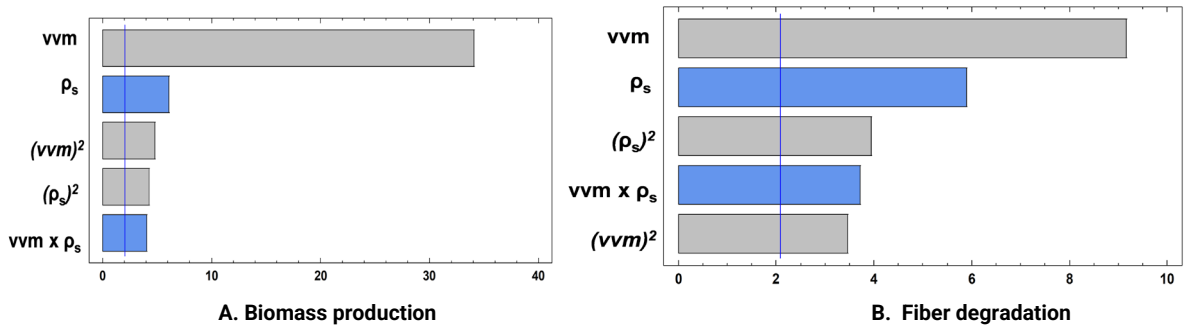


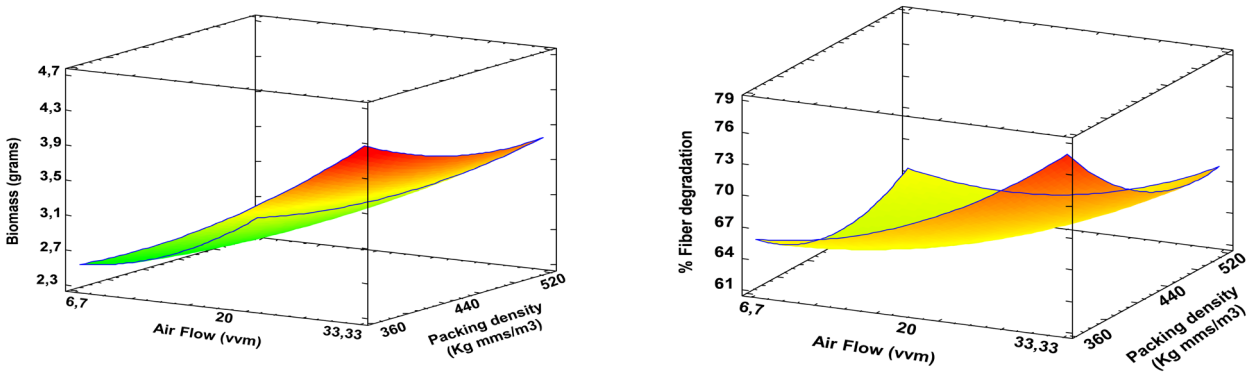
Fig. 3. Standardized Pareto diagram for the analysis of the statistical significance of the factors: A. biomass production in the different combinations of airflow vvm and packing density ρs and B. Fiber degradation.

2.4 Optimizing AN19bc growth in an SSF system

Figure 4 shows the combination of factors with which the highest biomass concentration is reached with air flow values of 33.33 vvm and ρs of 360 kg Mss/m^3. This demonstrates that the best biological performance of *A. niger* strain AN19bc in an SSF system occurs with high air flows and low packing densities. These results coincide with those reported by Gutarra et al [45] and Díaz et al [44] where they indicate that high volumes of airflow favor the production of biomass and extracellular enzymes. On the contrary, authors such as Vivekanand et al [48] reported that a high airflow negatively affects the production of laccase enzymes produced with *Aspergillus* fumigatus, Abdul and Webb [49] used flows of 4 vvm, a value with which they obtained good results.

The heat generated in SSF systems negatively affects fungal metabolism and possibly biomass and enzyme production [50]. This parameter is generally regulated by dosing saturated air [51]. However, Pérez et al [52] and Chávez et al [53] indicate that high air flows cause the drying of the packed bed, which in turn influences the mass transfer phenomena, which also affects the absorption of nutrients and the growth of the fungi used in the SSF systems. On this, Vivekanand et al [48] and Adinarayana et al [54] determined that the best production of laccase and lipase enzymes occurs when using an air saturation chamber to maintain humidity in the bed of SSF bioreactors.

The ρs is another important factor in an SSF system, studies carried out by Díaz et al [44] demonstrated that at low densities of the substrate, it favors the transport of oxygen and the growth of the fungus. Reyes et al [34] also state that the metabolism of *A. niger* is affected by the increase in glucose in the medium, causing osmotic stress in the microorganism, which leads to energy expenditure to survive in the environment.



Biomass *= 2.91 + 0.75(vvm) − 0.135 (ρ_s) + 0.185(vvm)^2 − 0.110(vvm x ρ_s) + 0.16 (ρ_s^2)*

%Fiber degradation *= 0.64 + 0.0396(vvm) − 0.026(ρ_s) + 0.026(vvm)^2 − 0.0198(vvm x ρ_s) + 0.0296(ρ_s^2)*

Fig. 4. Response surface analysis of biomass production and fiber degradation at different airflow conditions (vvm) and packing density (ρs) in an SSF system.

On the other hand, studies carried out by Ramírez [55] showed high lipase activity of 1505 Ug^{-1} and low of 42.77 Ug^{-1}, in 100 grams of coffee residue in an SSF system. Also, Costa et al [56] found that densities between 586 and 858 g/L of rice bran, it is beneficial for the expression of ligninolytic enzymes by *A. niger*, values that coincide with what was evaluated in the present investigation for the extraction of lipase in the AN19bc strain of *A niger*.

The effect of density is related to the interparticle space of the packed bed. And just like humidity and airflow, affect mass transfer phenomena, increased packing density makes the removal of metabolic heat more difficult, it increases the concentration of CO_2 in the fermenter and limits the growth of fungi [57]. The fungi can penetrate the fibers of the packed bed, which favors a greater production of biomass [58] and extracellular enzymes. However, at unsuitable high bed densities, this capacity is limited [59].

2.5 Change of composition of the residual

The bromatological composition of the sugarcane bagasse after the resulting SSF process is presented in Figure 5. Where an increase in the concentration of ash, protein, and lignin content can be observed, and a decrease in fiber content. These results are similar to the investigations carried out by Duru, Uma [60], and Oboh [61], as well as Pineda et al [62], indicating that the increase in protein is due to the

transformation of polymers into components such as CO_2 and H_2O, while nitrogen is concentrated and transformed to form part of the biomass of the fungus as they grow.

One of the most important changes was the one suffered by the fiber, which had a decrease in its concentration at the end of the fermentation process, studies carried out by Grandar et al [63], mention that acid lipase has phenolic structures, as is the case with lignin, and non-phenolic ones. For its part, it is known that lignin is not hydrolyzing and its presence limits the enzymatic hydrolysis of cellulose and hemicellulose, which can also influence an increase in ash concentration since silicon and other minerals accumulate on the surface of the fiber causing rigidity to the fiber limiting the penetration of enzymes and their degradation as mentioned [46].

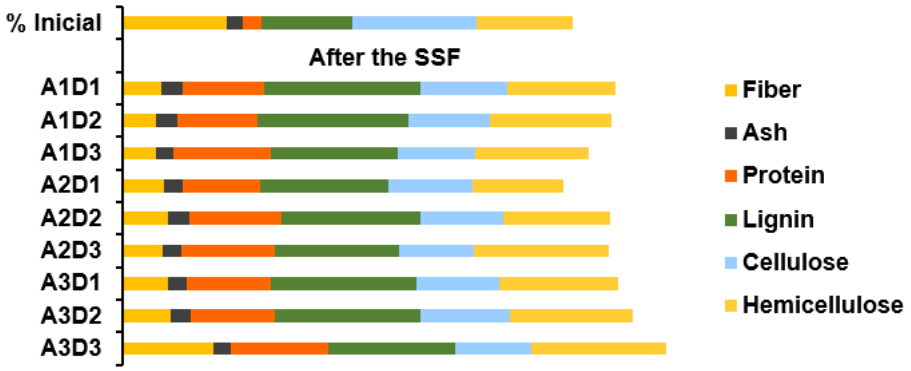


Fig. 5. Residual component concentration after SSF.

Studies reported by Hussein et al [64] and Karnitz et al [65] have shown that sugarcane bagasse can adsorb contaminants in water such as oils, in addition to separating heavy metals such as cadmium, copper, and lead. Also, microorganisms have been used to improve nutrient residues through an SSF system [47]. In the present investigation, it was possible to observe that the components of the residual are concentrated in the substrate, and also the percentage of fiber decreases, which could add value to this by-product that is obtained after the fermentation process, where it can be used as a substrate for agricultural soils [66].

3. Material and Methods

To obtain the fungal inocula, 6 isolates of *A. niger* strains obtained from coffee waste, sugarcane bagasse, and rice husks were used, where they were preserved in potato dextrose agar (PDA) culture medium in the laboratory of the Research Institute of the Technical University of Manabí. These strains were cooled and incubated in Labnet 311DS equipment for 14 days at a temperature of 28-30ºC, and then stored at -4ºC for subsequent analysis.

3.1 Determination of the strains with the highest lipase production

The strain with the highest lipase capacity was selected, using the 6 isolates, where they were sown in a 250 mL Erlenmeyer flask containing PDA medium and then incubated for seven days at 28-30ºC. To determine the enzymatic activity in each strain, the methodology used by Ponce et al [67] was used, where containing trace elements, previously sterilized in an autoclave for 20 minutes at 121ºC. This medium is incubated at 30ºC and shaken at 120 rpm for 30 minutes, then the liquid medium was filtered on a 125 mm filter paper and then on a 0.45 µm syringe filter to prevent the passage of hyphae. This filtered medium was stored at 4ºC for its subsequent enzymatic quantification analysis, which was determined by the method used by Nema et al [29].

3.2 Kinetics of the enzyme lipase in SmF

The strain with the highest lipase production was evaluated for 14 days, to determine the maximum production time. This strain was sown in a liquid medium containing a carbon source ($C_{12}H_{22}O_{11}$ 2g/L), a nitrogen source ($(NH_4)_2SO_4$ 10g/L], minerals [12 g/L of NaH_2PO_4; 0.3 g/L of $MgSO_4.7H_2O$, 0.25 g/L of $CaCl_2$ and 20 mL of trace elements) and inducer (2g/L of olive oil), to obtain the period with the highest enzyme production. The culture was developed in an incubator (Labnet 311DS) with a temperature of 30ºC at 100 rpm and these conditions were maintained during the evaluation time [68], where therefore the enzymatic activity was determined using the methodology of Nema et al [29].

3.3 Residual pre-treatment

Sugarcane bagasse is used as a support for the microorganism in the SSF system to produce the lipase enzyme. The residual was subjected to a previous treatment where it was washed with 1% NaClO, then rinsed with plenty of distilled water, and dried in an oven for 24 hours at 82ºC. The material was ground to reduce its size to a particle diameter of ≤ 20 mm, then it was enriched by a sterile nutrient medium, proposed by Dustet & Izquierdo [69], to adjust the C:N ratio that the microorganism needs for its development, it contained 8% p/p sss of $(NH_4)_2SO_4$; 2.37% p/p of NH_2CONH_2 and olive oil as inducing medium; the humidity of the medium was 70% with a pH of 4.5.

3.4 Solid State Fermentation System (SSF)

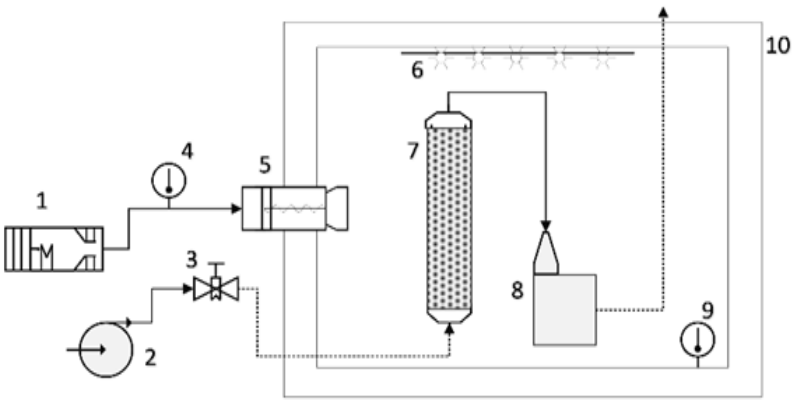
The experiment was carried out in cylindrical bioreactors with a volume of 150 mL, under the model and conditions published by Rosero and Dustet [70] (Figure 6).

3.5 Optimization of process parameters

3.5.1 *CO_2 and biomass generation evaluation*

The columns were connected to a trap with potassium hydroxide at 0.5 N, to trap the CO_2 produced in each of the columns by the provisions of the Ecuadorian Technical

Standard NTE INEN 2642:2012, this process was carried out for 21 days with data collection intervals of every 3 days. The production of CO_2 allowed us to calculate the growth curve of the microorganism in each treatment and, in addition, using mathematical expressions Doran [71], taking into account the stoichiometry of the reaction, the grams of biomass produced during the fermentation time were calculated.



1. Heat generation system (~220v).
2. Air nebulizer (10L min^{-1})
3. Air regulation valve.
4. Control thermometer.
5. Heat distribution plate.
6. Lighting system (max 2000Lux).
7. Solid fermentation column.
8. gas trap.
9. Internal control thermometer.
10. Structure of the fermentation module.

Figure 6. Design of bioreactors for the SSF process.

Table 2. Mathematical expressions and thermodynamic coefficients used in the calculation of the biomass generated in the SSF process.

Estimation of the biomass generated in each time interval	Estimation of the biomass-CO_2 yield coefficient
$X_t = t_n = X_{t_{n-1}} + \left[Y_{\frac{X}{CO_2}} * \Delta CO_2\right]$ Ec.1	$Y_{\frac{X}{CO_2}} = \frac{\frac{3\eta}{2\sigma_b \gamma_b}}{\frac{1.375(1-\frac{\gamma_s}{\gamma_b}\eta-\varepsilon p\frac{\gamma_s}{\gamma_b})}{\frac{\gamma_s}{4}}}$ Ec. 2
Thermal parameters of the fermentation system with *A. niger*	
Aspergillus niger	σ_b=0.454 γ_b= 4.09
Sugar cane bagasse	σ_b=0.420 γ_b= 4.09

Dustet et al., (2004).

3.5.2 Determination of substrate to biomass

The biomass/substrate yield (Yx/s) was determined, where Δx is the biomass produced and Δs is the amount of substrate consumed and is calculated using equation [72]:

$$Y_{X/S} = \frac{\Delta x}{\Delta s} \quad \text{Ec.3}$$

3.6 Statistical analysis

A multilevel factorial design (2^3) was applied where the effect of three levels of packing density (D1=360, D2=440, and D3=520 kg DMS/m^3) and three different dry air flows (A1=6.7; A2=20 and A3=33.3 vvm). The parameters, biomass production, and fiber degradation were analyzed, and the measurements were made in triplicate, taking samples over time.

The data were analyzed in the Statgraphics Centurion XVI software, where the adjustment curve in biomass production was determined, the same that allows for the calculation of the specific growth speed, latency time and amount of biomass, and optimal airflow.

4. Conclusions

The fungus *A. niger* AN19bc isolated from sugarcane bagasse reached a high production of lipase. In the kinetics of enzyme production, during day 11 it reached the highest point and is associated with the generation of fungal biomass. The effect of the factors evaluated on the airflow at a higher level had significance in the production of biomass for obtaining lipase enzyme, The best treatment in the present investigation was with an airflow of 33.33 vvm and a packing density of 360 kg DMS/m^3, resulting in values of 4.32 g DMS of biomass.

A. niger enriches the nutrient content of sugarcane bagasse, allowing it to concentrate protein and degrade fibers, in an SSF system, which, in addition to obtaining enzymes, could be an effective means to enrich lignocellulosic substrates and incorporate it back into the production chain as a substrate for agricultural soils.

However, the lipase enzyme, due to its ability to degrade complex organic compounds, makes it an interesting option for industries, since it allows cleaner and more environmentally friendly solutions to be offered to address pollution problems.

Author Contributions

Kayna Hidalgo Zambrano: Research, data analysis, project manager, draft writing. Carlos Delgado Villafuerte: Supervision, resources, editorial review. Ernesto Rosero Delgado: Research, supervision, data analysis, proofreading, and text editing.

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