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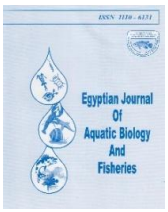


# Production of exopolysaccharides of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* to absorb lead in the sediment of aquaculture pool.

**Juan Diego Valenzuela-Cobos[1*], Ana Grijalva[2], Raúl Marcillo[3] and Fernanda Garcés[4]**

[1]Universidad Espíritu Santo - Ecuador
[2]Facultad de Ciencias Químicas, Universidad de Guayaquil - Ecuador
[3]Departamento de Oceanografía Naval. Instituto Oceanográfico de la Armada
[4]Facultad de Ingeniería, Escuela Ingeniería Ambiental. Universidad Nacional de Chimborazo. Km 1 / vía a Guano s/n, Riobamba, 060150. Ecuador

***Corresponding Author: juan_diegova@hotmail.com**

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**ABSTRACT**

*Colletotrichum gloeosporioides* and *Rhizopus stolonifer* were cultivated in two culture media: potato dextrose agar (PDA) to determine the maximum growth mycelial speed ($\mu_{max}$) and lag phase ($\lambda$) using the Gompertz and Hills mathematical models, also used liquid medium potato dextrose broth (PDB) to obtain production of mycelial biomass and exopolysaccharides (EPS). *Colletotrichum gloeosporioides* showed the highest maximum growth rate (0.38 $day^{-1}$) and the lowest lag phase duration (1.85 h) on PDA medium, Colletotrichum gloeosporioides cultivated on liquid medium presented the highest biomass production (76.15%) and exopolysaccharides production (80.00%). Exopolysaccharides (EPS) obtained from the liquid culture medium of *Colletotrichum gloeosporioides* presented the highest absorption content of lead (0.015 $mg.g^{-1}$). The results obtained that exopolysaccharides of *Colletotrichum gloeosporioides* cultivated on PDB medium showed the highest absorption of lead (Pb) in the sediment of the aquaculture pool.

## INTRODUCTION

Heavy metals are usually found in nature as components of the earth's crust, in the form of salts, minerals or other compounds, these elements are presented in high concentrations result in cytotoxic and lethal effects, even in small concentrations such as Pb, Cd, Co, Ni, Zn and Cu **(Nies, 1999; Lau *et al*., 2005; Pérez *et al*., 2008**). In the last decade, anthropogenic impacts have released a great pollution to the environment, heavy metal pollution presents a problem growing world (**Garbisu and Alkorta, 2003**). Toxicity to these elements in humans occurs through the blockage of the various biological activities, such as enzymatic inactivation by the formation of bonds between the metal and hydrogen sulfide groups (-SH), altering various functional groups of

proteins resulting in irreversible damage by modifying the formation of biological molecules (**Garbisu and Alkorta, 2003; Rajendran *et al*., 2003**).

The heavy metals pollution is the main challenge in the environmental field because they have toxic or poisonous characteristics (**Lucho *et al*., 2005**). They have been shown different technologies to solve this problem, such as: the use of microorganism in the treatment of wastewater contaminated with heavy metals and in recovery of metals in mining wastes or metallurgical effluents (**Gadd, 1997**). Microorganisms are also used as they modify the concentration of heavy metals in the environment thanks to the action of enzymatic and non-enzymatic mechanisms to remove metals in solution (**Rajendran *et al*., 2003**), also there is other method with more efficiency such as the adsorption of heavy metals by exopolysaccharides (**Pagnanelli *et al*., 2000**).

Exopolysaccharides (EPS) are complex group of macromolecules of carbohydrates, these are secreted by bacteria, fungi and algae, which accumulate extracellularly giving a mucilaginous appearance (**Comte *et al*., 2006**). In bioremediation methods, exopolysaccharides use functional groups ionizable such as carboxyl, acetate, hydroxyl, amine, phosphate or sulfate groups that they act as active sites for ionic biosorption (**Deschatre *et al*., 2013**).

The aim of this research was to determinate the mycelial kinetics of the phytopatogenic strains on PDA dishes, the production of biomass and exopolysaccharides in liquid media (PDB) and the adsorption of lead (Pb) in the sediment of aquaculture pool by the exopolysaccharides produced of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

## MATERIALS AND METHODS

### Biological material

In this research was used the phytopatogenic fungi: *Colletotrichum gloeosporioides* (XY001) and *Rhizopus stolonifer* (CS005). Stocks of all strains are deposited at the fungal collection of Research and Development Laboratory of Ecuahidrolizados.

### Culture media

The culture media was prepared by dissolving 18 g of potato dextrose agar (PDA) in 0.50 L of distilled water using an Erlenmeyer flask. The flask was autoclaved at 121 °C for 30 min, subsequent, 10 mL of sterile medium were poured into Petri dishes. The dishes with the medium solidified were put in plastic bags and incubated at 28 °C for 24 h to check the sterility (**Eger *et al*., 1976**).

### Preparation of liquid culture

The liquid culture was prepared by dissolving 39 g of PDB potato dextrose broth in 1 L of distilled water using an Erlenmeyer flask. The flask was sterilized in an autoclave at 15 psi (121 ° C) for 15 minutes.

**Mycelial kinetics**

The mathematical models of Gompertz and Hills were used to determine the mycelial growth kinetics of phytopathogenic fungi such as: *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* on papa dextrose agar medium (PDA) (**Liu *et al*., 2017**):

log N = A + C * exp {-exp [-B (t-M)]}, where:

A, B, C = model parameters

t = days of mycelial colonization

M = day with maximum growth rate

log N = growth kinetics

Using the model parameters was calculated the maximum growth rate ($\mu_{max}$) see Eq. (1) and the lag phase ($\lambda$) see Eq. (2) (**Gibson *et al*., 1987; Hills and Wright, 1994**):

**Eq. (1)** $\mu_{max}$ = (B * C) / e, where: e = Euler constant

**Eq. (2)** $\lambda$ = [ln (1+ ($\mu_{max}$ / v)] / $\mu_{max}$, where: v = $\mu_{max}$

**Biomass production**

Mycelium of phytopathogenic fungi such as: *Colletotrichum gloeosporioides* (XY001) and *Rhizopus stolonifer* (CS005) was activated on PDA plates at 25 ℃ for 5 days. Two discs with a size of 5.5 mm were cut from the edge of the mycelium on PDA, and were inoculated in an Erlenmeyer flask with 100 mL of the liquid medium. The flask with the medium was put in a shaking incubator at 200 rpm for 6 days at 25 $^{o}$C. Finally, the biomass was filtered through Whatman # 1 filter paper and dried to constant weight. (**Lakzian *et al*., 2008; Taskin *et al*., 2012**).

**Exopolysaccharides production**

The culture broth and the water used to wash the sieve biomass were filtered through Whatman # 1 filter paper, and was added 150 mL of ethanol to precipitate the polysaccharides. Finally, the precipitate EPS that was filtered, dried to constant weight at 40 °C (**Rasulov *et al*., 2013**).

**Biosorption studies**

Batch studies were perfomed using 100 mg of exopolysaccharides in 250 mL Erlenmeyer shaker flash containing 100 mL of 10-100 ppm metal solution (adjusted to pH 6.0) at 25 $^{o}$C and 160 rpm in a shaking incubator for 15 min. Beads of biomass were centrifuged, the supernatant was decanted, filtered and metals ions were then analyzed by ion exchange chromatography (**Xie *et al*., 1996**).

Isothermal adsorption of lead was studied by using Langmuir model. The Langmuir equation is valid for monolayer sorption onto a surface with a finite number of identical sites, see Eq. (3).

**Eq.(3)** $q_{eq}=(Q^o\ bC_{eq})/(1+\ bC_{eq}\ )$

where: $Q^o$ is the maximum amount of the metal ion per unit weight of cell to form a complete monolayer on the surface bound at high Ceq ($mg.g^{-1}$) and b is a constant related to the affinity of the binding sites.

**Statistical analysis**

In all analyzes, a completely randomized design and the results were studied using one-way analysis of variance (ANOVA) to determine the significance of individual differences at $p<0.05$ level, the specific maximum growth rate ($\mu_{max}$), the lag phase ($\lambda$), the biomass content and exopolysaccharides produced by the submerged liquid used in cultivation of phytopatogenic fungi, when statistical differences were found, the Duncan Test with $\alpha = 0.05$ was applied. The analyses were carried out using statistical software (Statgraphic ver. 16).

## RESULTS

**Mycelial growth**

Table 1 shows the mycelial kinetics of the phytopathogenic fungi *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* on PDA medium using the mathematical models of Gompertz and Hills (**Valenzuela-Cobos *et al.*, 2017**).

The average of parameter A was -28.29, while B was 1.12 and for C it was 76.87. **Regina (2001)** determined the volume of mycelial growth for two strains of *Lentinula edodes* where values of A ranged from 1.57 to 16.13, values of B since 28.36 to 86.53 and values of C between 11.89 and 44.24 were obtained. A is the parameter that indicates the maximum potential of the growth rate, while B is the biological variable that relates the growth rate and the incubation time, and C is the biological parameter that correlates the incubation time and the speed snapshot (**Fekedulegn *et al.*, 1999**).

*Colletotrichum gloeosporioides* and *Rhizopus stolonifer* presented specific growth speeds between 0.28 and 0.38 $day^{-1}$, and the latency phase since 1.85 to 2.40 h. using the Gompertz and Hills models, these models describe growth trends in terms of static accuracy and simplicity (**McDonald and Sun, 1999**). **Guillén-Navarro *et al.* (1998**) reported $\mu_{max}$ values of 0.86 $day^{-1}$ on day $12^{th}$ from *Pleurotus ostreatus* cultivated on agar DLA, and also presented the $\mu_{max}$ value of 0.65 $day^{-1}$ of the same strain on synthetic medium with yeast extract and glucose concentration of 2.5 (g/L). The specific growth rate ($\mu_{max}$) indicates the ability of the strain to absorb nutrients in both media (**Liu *et al.*, 2017**). **Straatsma *et al.* (1991)** reported $\lambda$ values for *A. bisporus* ranged from 0.46 to 0.71 h through the use of logistic functions. The lag phase ($\lambda$) indicates the strain's ability to adapt to new environmental conditions (**Chatterjee *et al.*, 2015**).

**Table 1.** Mycelial kinetics of *Colletotrichum gloeosporioides* and *R. stolonifer*.

| Strains | A | B | C | e | $\mu_{max}$ ($day^{-1}$) | λ (h) |
|---|---|---|---|---|---|---|
| *Colletotrichum gloeosporioides* (XY001) | -4.92 | 0.02 | 13.31 | 2.718 | $0.38^{A}$ | $1.85^{b}$ |
| *Rhizopus stolonifer* (CS005) | -51.68 | 2.22 | 140.43 | | $0.28^{B}$ | $2.40^{a}$ |

*A, B and C= Parameters of the Gompertz model, e= Euler constant.

*Uppercase letters indicate difference between the specific growth speed of the phytopatogenic fungi, while lowercase letters indicate difference between the lag phase of the phytopatogenic fungi according to Duncan's test ($p < 0.05$), n = 10.

**Production of biomass and exopolysaccharides**

Table 2 shows the production of biomass from the submerged liquid of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

Biomass production by *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* fungi were between 56.25% and 76.17%. **Taskin *et al.* (2012)** used liquid culture composed of glucose (40 g/L), yeast extract (3 g/L) and tryptone peptone (10 g/L) for the growth of the strain of *Morchella esculenta* obtaining 16% of biomass.

**Table 2.** Biomass production on PDB medium of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

| Strains | Wet weight (g) | Dry weight (g) | Biomass (%) |
|---|---|---|---|
| *Colletotrichum gloeosporioides* (XY001) | 0.80 | 0.40 | $76.15^{a}$ |
| *Rhizopus stolonifer* (CS005) | 0.90 | 0.70 | $56.25^{b}$ |

* Different letters in each column indicate significant difference between the biomass produced by two strains of phytopathogenic fungi with level $p < 0.05$, according to the Duncan test, n = 10.

Table 3 presents the production of exopolysaccharides from the submerged liquid of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*. The production of exopolysaccharides from the fungi *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* were between 15% and 80%. **Taskin *et al.* (2012)** used liquid culture

composed of glucose (40 g/L), yeast extract (3 g/L) and tryptone peptone (10 g/L) for the strain of *Morchella esculenta* presenting production of exopolysaccharides (4.80%). Submerged cultivation represents an alternative form of rapid and efficient production of mycelial biomass and exopolysaccharides (**Confortin *et al*., 2008**). Peptons represent not only a source of organic nitrogen but also a source of specific amino acids or peptides. These are defined as protein hydrolysates that are easily soluble in water and are not precipitable by heat, by alkali or by saturation with ammonium sulfate.

**Table 3.** Production of exopolysaccharides in submerged medium PDB of fungi *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

| Strains | Exopolysaccharides (%) |
|---|---|
| *Colletotrichum gloeosporioides* (XY001) | 80.00[a] |
| *Rhizopus stolonifer* (CS005) | 15.00[b] |

* Different letters in each column indicate significant difference between the production of the exopolysaccharides of the two strains of phytopathogenic fungi with level $p < 0.05$, according to the Duncan test, n = 10.

**Langmuir adsorption isotherms**

Using the Langmuir model, maximum adsorption of lead was estimated to be 0.015 $mg.g^{-1}$ in the sediment of aquaculture pool by the exopolysaccharides produced from the submerged liquid used in cultivation of *Colletotrichum gloeosporioides*. For otherwise, exopolysaccharides obtained by the submerged liquid (LC3) used in the production of *Rhizopus stolonifer* showed adsorption values of lead of 0.009 $mg.g^{-1}$ in the sediment of aquaculture pool (Table 4). **Lakzian *et al.* (2008)** reported optimal conditions for lead(II) biosorption by *Arthrobacter* sp. 25 (free-living cells) from aqueous solution were an initial lead(II) concentration of 108.79 mg/L, pH of 5.75, and a biosorbent dose of 9.9 g/L. The predicted maximum theoretical adsorption capacity of lead(II) was 9.88 mg/g. The adsorption capacity increased with the atomic mass of the elements (**Tobin *et al*., 1984**). The adsorption of the heavy metals is related with the medium used in cultivation of the strains.

**Table 4.** Langmuir isotherm model constants for adsorption of lead by EPS produced from submerged culture of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*.

| Fungi | $Q^o$ ($mg.g^{-1}$) | $R^2$ |
|---|---|---|
| *Colletotrichum gloeosporioides* (XY001) | 0.015 | 0.9967 |
| *Rhizopus stolonifer* (CS005) | 0.009 | 0.9901 |

## CONCLUSION

The Gompertz and Hill models showed that *Colletotrichum gloeosporioides* showed the highest maximum growth specific speed ($\mu_{max}$) and shorter phase duration ($\lambda$) on PDA medium.

The liquid culture used in the cultivation of *Colletotrichum gloeosporioides* presented the highest production of biomass and exopolysaccharides. The exopolysaccharides produced from the liquid culture of *Colletotrichum gloeosporioides* showed the highest adsorption of lead in in the sediment of aquaculture pool in comparison with the exopolysaccharides obtained from the submerged culture of *Rhizopus stolonifer*.

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