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Original paper

Sorption of Zinc by exopolysaccharides produced by liquid media of phytopathogenic fungi

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Abstract

Phytopathogenic fungi such as: *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* were cultivated in three different liquid culture media: LCC (glucose 40 g L⁻¹, yeast extract 3 g L⁻¹), LC2 (glucose 40 g L⁻¹, yeast extract 3 g L⁻¹ and tryptone peptone 2 g L⁻¹) and LC3 (glucose 40 g L⁻¹, yeast extract 3 g L⁻¹ and tryptone peptone 10 g L⁻¹) under pH of 5.5 for the production of mycelial biomass and exopolysaccharides (EPS). The liquid culture medium (LC3) used in cultivation of *Colletotrichum gloeosporioides* showed the highest production of biomass (15.40 g L⁻¹) and exopolysaccharides (3.40 g L⁻¹). Exopolysaccharides (EPS) obtained from the liquid culture medium (LC3) of *Colletotrichum gloeosporioides* presented the highest absorption content of Zinc (56 mg g⁻¹). The results presented that the exopolysaccharides (EPS) produced by *Colletotrichum gloeosporioides* showed the greatest biosorbent capacity of Zinc (Zn) using the culture medium with the highest amount of tryptone peptone.

Keywords

Biomass, exopolysaccharides, liquid culture medium, phytopathogenic fungi.

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Introduction

Colletotrichum sp. and *Rhizopus* sp. are phytopathogenic fungi that are used to improve the biodegradation of organic wastes like: mangoes and oranges for being used like compost (MENA-NEVAREZ & al [19]; VALENZUELA-COBOS & al [29]). Composting is defined as the biological decomposition of organic matter under controlled aerobic conditions to form a stable, humus-like end product (JARA-SAMANIEGO & al [13]). The main problem of compost is the high content of heavy metals that cause an increase in soil pollution, and has a directly relation in the toxicity of the food cultivated (CABRERA & al [3]).

Heavy metals usually form compounds that can be toxic, carcinogenic or mutagenic, even in very low concentrations such as cadmium (Cd), copper (Cu), mercury (Hg), plumb (Pb), and Zinc (Zn) (LAU & al [15]; PÉREZ & al [23]). Zinc is one of the metals with more discharge in the environment, the recommended upper limit for discharge is about 5 mg L⁻¹ (PURANIK & PAKNIKAR [24]). Bioremediation of heavy metal pollution is the major challenge in the environmental field (VOLESKY [32]), different technologies have been proved to solve this problem such as: the use of biomass of microorganisms in the treatment of heavy metal-contaminated wastewaters, in the recovery of metals in mining wastes or in metallurgical effluents (LORENZ [18]; GADD [11]), and the extracellular polysaccharides (EPS) secreted by bacteria, fungi and algae are recommended as surface active agents for heavy metal removal, the second method has showed the highest adsorption capacity of heavy metals in comparison with the first technique (VEGLIO & al [31]; PAGNANELLI & al [15]).

The adsorption of heavy metal by EPS is a metabolism-independent process, and it is attributed to interaction between metal cations and negative charges of acidic functional groups of EPS (RASULOV & al [25]). The interaction has stimulated specific research interest due to its important ecological and practical implications (PAPERI & al [21]). This method of bioremediation uses the EPS present ionisable functional groups such as carboxyl, acetate, hydroxyl, amine, phosphate or sulfate groups which may act as active sites for ionic biosorption (COMTE & al [6]; DESCHATRE & al [8]). Submerged liquid is the most common culture used in the production of biomass and exopolysaccharides, this culture medium offers potential advantages of increased mycelial production in shorter time without significant contamination problem (BAE & al [1]; KIM & al [14]). However, there are not studies about the use of exopolysaccharides produced from liquid media of phytopathogenic fungi such as: *Colletotrichum* sp. and *Rhizopus* sp. to removal Zinc.

The aim of this research was determined the biomass and the exopolysaccharides (EPS) production from the three different liquid cultures used in the cultivation of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*, the sugar composition of the exopolysaccharides and

the adsorption capacity of Zinc in aqueous solution by the exopolysaccharides (EPS) of the phytopathogenic fungi.

Materials and Methods

1. Biological material

In this experiment was used the following phytopathogenic fungi: *Colletotrichum gloeosporioides* (CL005) and *Rhizopus stolonifer* (RH015). The strains are maintained on PDA dishes. Stocks of all strains are deposited at the fungal collection of Research and Development Laboratory of Ecuahidroliizados.

2. Culture media

The culture media was prepared by dissolving 39 g of potato dextrose agar (PDA) in 1 L of distilled water using an Erlenmeyer flask. The flask was sterilized in autoclave at 15 psi (121°C) for 15 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. Then, the Petri dishes without contamination were used for propagation of the mycelium of phytopathogenic fungi (EGER & al [10]; COELLO-LOOR & al [5]).

3. Preparation of liquid culture

Liquide Culture Control (LCC): 1 L of distilled water with glucose (40 g L⁻¹), yeast extract (3 g L⁻¹).

Liquide Culture 2 (LC2): 1 L of distilled water with glucose (40 g L⁻¹), yeast extract (3 g L⁻¹) and tryptone peptone (TP) 2 g L⁻¹.

Liquide Culture 3 (LC3): 1 L of distilled water with glucose (40 g L⁻¹), yeast extract (3 g L⁻¹) and tryptone peptone (TP) 10 g L⁻¹.

The medium pH was adjusted to 5.5 by addition of either 1N NaOH or 1N HCl (TASKIN & al [27]).

4. Biomass production

Mycelial of phytopathogenic fungi: *Colletotrichum gloeosporioides* (CL005) and *Rhizopus stolonifer* (RH015) were activated by culturing at 25°C for 6 days on PDA dishes. Two discs with 5.5 mm size, cut from the edge of the mycelia on PDA, were then inoculated in 100 mL of the three solutions of liquid culture. All production studies were carried out at 25°C and 200 rpm in a shaking incubator for 6 days. Cellular biomasses were separated by using a 17000 rpm centrifuge at 4°C, then was washed from the sieve with distilled water, filtered through Whatman #1 filter paper, and dried to constant weight at 70°C (LAKZIAN & al [17]).

5. Exopolysaccharides production

The culture broth and the water used to wash the biomass off the sieves were filtered through Whatman #1 filter paper and evaporated to 50 mL under reduced pressure at 80°C. This reduced volume was added to 150 mL of ethanol, in order to precipitate the polysaccharides. Finally, the precipitate exopolysaccharides (EPS) were filtered out, dried to constant weight at 40°C (WAGNER & al [33]; RASULOV & al [25]).

6. Determination of optimal TP concentration

The optimal concentration of tryptone peptone (TP) in the composition of the liquid culture was necessary the biomass content and exopolysaccharides obtained with the different liquid culture, see Eq. (1) – (2).

$$(1) \quad \%BCI = \frac{100 \times P_{BC}}{C_{BC}} - 100$$

$$(2) \quad \%EPSCI = \frac{100 \times P_{EPSC}}{C_{EPSC}} - 100$$

where: %BCI is the increase in the biomass concentration, P_{BC} is the biomass concentration at any concentration of TP, C_{BC} is the biomass concentration in the control medium, %EPSCI is the increase in the EPS concentration, P_{EPSC} is the EPS concentration at any concentration of TP and C_{EPSC} is the EPS concentration in the control medium (TASKIN & al [27]).

7. Carbohydrate composition of EPS

The carbohydrates were determined by phenol sulfuric acid method using glucose as the standard. Sugar composition was analyzed by gas chromatography with a fused silica capillary column (300 mm × 0.25 mm) and a flame ionization detector. Total protein was determined by the Lowry method with bovine serum albumin as the standard (HWANG & al [12]).

8. Biosorption studies

Batch studies were performed using 100 mg of exopolysaccharides in 250 mL Erlenmeyer shaker flask containing 100 mL of 10-100 ppm metal solution (adjusted to pH 6.0) at 25°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 & al [34]).

Isothermal adsorption of zinc 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).

$$(3) \quad q_{eq} = \frac{Q^0 b C_{eq}}{1 + b C_{eq}}$$

where: q_{eq} is the adsorption capacity at equilibrium, Q^0 is the maximum adsorption capacity corresponding to monolayer coverage, C_{eq} is the residual adsorbate concentration at equilibrium in solution and b is the Langmuir constant correlated to the adsorption energy.

9. 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 biomass content and exopolysaccharides produced by the submerged liquid used in cultivation of phytopathogenic 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 and Discussion

1. Biomass and exopolysaccharides production

Table 1 shows the production of biomass and exopolysaccharides from the submerged liquid of *Colletotrichum gloeosporioides*.

The liquid culture (LC3) used in the cultivation of *Colletotrichum gloeosporioides* presented 45% more biomass content in relation with the solution without tryptone peptone (LCC), while using the solution (LC2) showed 20% more biomass content in relation with solution without tryptone peptone (LCC). For otherwise, the solution (LC3) used in the production of *Colletotrichum gloeosporioides* presented 79% more exopolysaccharides in relation with solution without tryptone peptone (LCC), while using the solution (LC2) showed 32% more exopolysaccharides in relation with solution without tryptone peptone (LCC). Similar results have been reported, Taskin & al [27] reported from the liquid culture composed of glucose (40 g L⁻¹), yeast extract (3 g L⁻¹) and tryptone peptone (10 g L⁻¹) of *Morchella esculenta*: biomass content of 16 g L⁻¹ and exopolysaccharides production of 4.80 g L⁻¹.

Table 1. Effect of various concentrations of tryptone peptone (TP) on biomass and exopolysaccharides production from *Colletotrichum gloeosporioides*

Medium	BC (g L ⁻¹)	BCI (%)	EPSC (g L ⁻¹)	EPSCI (%)
Liquide Culture Control (LCC)	10.60±0.07 ^b	-	1.90±0.34 ^c	-
Liquide Culture 2 (LC2)	12.70±0.53 ^b	20	2.50±1.01 ^b	32
Liquide Culture 3 (LC3)	15.40±0.18 ^a	45	3.40±0.06 ^a	79

**LCC: 1 L of distilled water with glucose (40 g L⁻¹), yeast extract (3 g L⁻¹), LC2: 1 L of distilled water with glucose (40 g L⁻¹), yeast extract (3 g L⁻¹) and tryptone peptone (TP) 2 g L⁻¹, LC3: 1 L of distilled water with glucose (40 g L⁻¹), yeast extract (3 g L⁻¹) and tryptone peptone (TP) 10 g L⁻¹.

*BC: biomass concentration, %BCI: increase in the biomass concentration, EPS: exopolysaccharides production, %EPSCI: increase in the EPS concentration.

*Different letters in each column indicated significant difference among the biomass and exopolysaccharides concentration produced by three culture media used in the cultivation of *Colletotrichum gloeosporioides* at level $p < 0.05$, according to Duncan's test, $n = 3$.

The production of biomass and exopolysaccharides from the submerged liquid of *Rhizopus stolonifer* is presented in the Table 2.

The liquid culture (LC3) used in the production of *Rhizopus stolonifer* showed 39% more biomass content in relation with the solution without tryptone peptone (LCC), whereas with the solution (LC2) showed 21% more biomass content in relation with the solution (LCC). On the other hand, the solution (LC3) used in the cultivation of *Rhizopus stolonifer* presented 64% more exopolysaccharides production in relation with solution without tryptone peptone (LCC), while using the solution (LC2) showed 27% more exopolysaccharides in relation with the solution without tryptone peptone (LCC). The submerged culture represents an alternative form of fast and efficient production of mycelial biomass and

exopolysaccharides (CONFORTIN & al [7]). Medium with nitrogen source tends to be the most expensive. Peptones represent not only a source of organic nitrogen but also a source of amino acids or specific peptides. They are defined as protein hydrolysates that are readily soluble in water and are not precipitable by heat, by alkalis or by saturation with ammonium sulphate. The most common peptones used for microbiological studies are bacto-peptone, tryptone peptone (TP), fish peptone (FP), meat peptone, neopeptone and protease peptone (PARRADO & al [22]; DUFOSSÉ & al [9]; TASKIN & ERDAL [26]; VASILEVA-TONKOVA & al [30]). The use of source of organic nitrogen such as: tryptone-peptone in the medium is directly related with the high production of biomass and exopolysaccharides.

Table 2. Effect of various concentrations of tryptone peptone (TP) on biomass and exopolysaccharides production from *Rhizopus stolonifer*

Medium	BC (g L ⁻¹)	BCI (%)	EPSC (g L ⁻¹)	EPSCI (%)
Liquide Culture Control (LCC)	8.50±0.21 ^b	-	1.10±0.09 ^c	-
Liquide Culture 2 (LC2)	10.30±0.04 ^b	21	1.40±0.27 ^b	27
Liquide Culture 3 (LC3)	11.80±0.07 ^a	39	1.80±0.53 ^a	64

*LCC: 1 L of distilled water with glucose (40 g L⁻¹), yeast extract (3 g L⁻¹), LC2: 1 L of distilled water with glucose (40 g L⁻¹), yeast extract (3 g L⁻¹) and tryptone peptone (TP) 2 g L⁻¹, LC3: 1 L of distilled water with glucose (40 g L⁻¹), yeast extract (3 g L⁻¹) and tryptone peptone (TP) 10 g L⁻¹.

*BC: biomass concentration, %BCI: increase in the biomass concentration, EPS: exopolysaccharides production, %EPSCI: increase in the EPS concentration.

*Different letters in each column indicated significant difference among the biomass and exopolysaccharides concentration produced by three culture media used in cultivation of *Rhizopus stolonifer* at level p<0.05, according to Duncan's test, n = 3.

2. Carbohydrate composition

The sugar composition only was determined to the exopolysaccharides produced from the submerged liquid (LC3) used in cultivation of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* (Table 3).

The sugar with more presence in the exopolysaccharides was the glucose with values of 34.72% from LC3 of *Colletotrichum gloeosporioides* and 70.58% from

LC3 of *Rhizopus stolonifer*. LEE & al [16] pointed out that different carbon source altered slightly the carbohydrate composition in polysaccharides. Each strain requires different source of nutrients such as: glucose and nitrogen sources for maximum mycelial growth or exopolysaccharides production (CHI & al [4]). The environmental conditions for mycelial growth and exopolysaccharides (EPS) production in liquid cultures are dependent on strains.

Table 3. Carbohydrate composition of EPS (Cg- LC3) and EPS (Rs- LC3) produced from submerged culture (LC3) of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*

Carbohydrates	Composition %	
	EPS (Cg- LC3)	EPS (Rs- LC3)
Fucose	nd	nd
Ribose	10.59	7.41
Arabinose	nd	nd
Xylose	nd	nd
Mannose	37.19	12.94
Galactose	18.10	9.07
Glucose	34.12	70.58

*EPS (Cg- LC3): exopolysaccharides obtained from Liquide Culture 3 (LC3) of *Colletotrichum gloeosporioides*, EPS (RS- LC3): exopolysaccharides obtained from Liquide Culture 3 (LC3) of *Rhizopus stolonifer*.

*nd: not detected

3. Langmuir adsorption isotherms

By using the Langmuir model, maximum adsorption of Zinc was estimated to be 56 mg g⁻¹ by the exopolysaccharides produced from the submerged liquid (LC3) 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 Zinc of 27 mg g⁻¹ (Table 4). LAKZIAN & al [17] reported values of adsorption of Zinc of 98 mg g⁻¹ obtained by the exopolysaccharides

produced from yeast extract mannitol broth (YEMB) culture medium used in cultivation of *Ensifer meliloti*, while (BAYRAMOGLU & ARICA [2]) presented values of adsorption of Zinc of 63.30 mg g⁻¹ obtained by the exopolysaccharides produced from subculturing on malt dextrose agar slants used in cultivation of *Lentinula edodes*. The adsorption capacity increased with the atomic mass of the elements (TOBIN & al [28]). 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 Zinc by EPS (Cg- LC3) and EPS (Rs- LC3) produced from submerged culture (LC3) of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*

Fungi	Q ⁰ (mg g ⁻¹)	b (L mg ⁻¹)	R ²
<i>Colletotrichum gloeosporioides</i> (CL005)	56	0.034	0.9752
<i>Rhizopus stolonifera</i> (RH015)	27	0.017	0.9807

Conclusions

The liquid culture (LC3) under pH of 5.5 used in the cultivation of *Colletotrichum gloeosporioides* presented the highest production of biomass and exopolysaccharides.

The exopolysaccharides produced from the liquid culture (LC3) of *Colletotrichum gloeosporioides* showed the highest adsorption of Zinc in comparison with the exopolysaccharides obtained from the submerged culture (LC3) of *Rhizopus stolonifer*.

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